European Journal of Cancer

22nd Biennial Congress of the European Association for Cancer Research

7–10 July 2012
Barcelona, Spain

Proceedings Book
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European Journal of Cancer

Aims and Scope

The European Journal of Cancer (including EJC Supplements) is an international comprehensive oncology journal that publishes original research, editorial comments, review articles and news on basic and preclinical research, clinical oncology (medical, paediatric, radiation, surgical), translational oncology and on cancer epidemiology and prevention.

For a full and complete Guide for Authors, please go to http://www.elsevier.com/locate/ejca
SAVE THE DATE

Join us in Munich for the 23rd Biennial Congress of the
EUROPEAN ASSOCIATION FOR CANCER RESEARCH

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www.ecco-org.eu
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Acknowledgements

The European Association for Cancer Research would like to acknowledge the generous ongoing support of its Sustaining Members.

And express sincere thanks for the generous support of the organisations sponsoring Symposia, Keynote and Award Lectures.

EACR also wishes to thank the following companies and organisations for their support of the Congress by taking part in the exhibition.

Abcam
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Toray Industries Inc.
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And Affymetrix, Agilent, ecancer and Illumina for additionally providing satellite symposia.

Official Media Partners

On behalf of the EACR-22 Congress Committees, we would like to acknowledge the collaboration and support of our official media partners:
Letter of Welcome

On behalf of the Executive Scientific Committee and the National Organising Committee we are very pleased to welcome you to Barcelona for the 22nd Biennial EACR Congress.

We are delighted that the Congress is actively supported by the Spanish Association for Cancer Research (ASEICA) and to be holding the Congress in one of Europe’s most vibrant cities, a city with a remarkable reputation for cancer research and cancer care.

EACR congresses are well known for providing a rich and varied programme. Over the next four days EACR-22 will provide a unique opportunity for participants to learn from and meet with leaders in the field of basic and translational research, apply new data to inform practice and ultimately improve patient outcomes.

The Congress will focus on the latest developments in basic and discovery driven translational research, through to personalised cancer treatment. Keynote and Award Lectures are complemented with focussed symposia across eighteen topics, and each morning begins with an opportunity to ‘Meet the Expert’ followed by Educational Lectures.

Posters will be displayed for review and ‘defence’ throughout the Congress in the Exhibition Hall and all participants are invited to assist in their evaluation. We hope that you will actively participate in the Poster Sessions and make your recommendations to the panel of judges.

We trust that you will return from the Congress inspired by colleagues from around the world and that you will have made new friends and scientific contacts that will support you in your essential work.

We are delighted to be welcoming you at what promises to be another highly educational, collaborative and successful EACR congress.

Julio E. Celis
EACR-22 Congress and Scientific Chair

Joan Seoane
National Organising Committee Chair

Congress Committees

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J. Navas
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L. Serrano
Accreditation Information

EACR-22 has been accredited by ACOE and the UEMS/EACCME

*What does it mean for you?*

The Accreditation Council of Oncology in Europe (ACOE) accreditation label provides delegates with a guarantee of a high quality and unbiased educational activity. ACOE accredited events are awarded an agreed number of CME credit points (1 CME credit per educational hour, 3 CME credits for a half-day, with a maximum of 6 credits per day).

EACR-22 has been granted European endorsement by the UEMS and awarded a **maximum of 18 hours of European Continuing Medical Education Credits (ECMEC's)**. The congress operates an honour system implying that each participant is expected to claim only those hours of credit that he/she actually spent at the congress (with a maximum of 6 credits per day, 3 for a half-day).

ACOE works in conjunction with the European Accreditation Council for Continuing Medical Education (EACCME) operating under the umbrella of the European Union of Medical Specialists (UEMS). EACCME acts as a clearinghouse, facilitating the accreditation endorsement by the national CME regulatory bodies.

CME credits gained through participation at EACR-22 are recognised by most national CME authorities in Europe, which have agreed to cooperate in this European system. Through a mutual agreement between the UEMS and the American Medical Association (AMA), European CME credits are also recognised towards the Physician's Recognition Award (PRA). To convert European CME credits to AMA PRA Category 1 Credits please contact AMA at: www.ama-assn.org.

Certificates of Attendance recording CME credits will be available online immediately after the Congress. You will receive an email link to a short questionnaire which also provides the link for you to print your Certificate of Attendance.

We kindly ask you to keep your Congress badge as you will need the unique badge code to print your Certificate of Attendance.

The Congress Secretariat will not mail Certificates of Attendance to participants after the Congress.

www.acoe.be
General Information

EACR-22 Congress Secretariat
c/o ECCO – the European CanCer Organisation
E-mail: EACR22@ecco-org.eu
www.ecco-org.eu/eacr22
During the congress, the Secretariat can be reached at +34 93 230 88 21

Congress Venue
Centre Convencions International Barcelona (CCIB) – FORUM
Rambla Prim, 1 – 17
08019 Barcelona, Spain
Tel: +34 93 230 10 00
www.ccib.es

Badges
For security reasons, delegates are requested to wear their badge at all times during the congress. Delegates having lost their badge can obtain a new one at the registration desk. A replacement fee of 50 EUR per participant will be charged.

Catering
Coffee Breaks: Coffee breaks courtesy of the organisers have been scheduled as follows:
Saturday 7 July: 15:00–15:30  Monday 9 July: 09:45–10:15
Sunday 8 July: 09:45–10:15  16:00–16:30  Tuesday 10 July: 09:45–10:15
Coffee breaks will take place in the exhibition hall, except on Saturday, when refreshments will be served in the foyer areas outside the session halls on the first floor.
Lunches: A complimentary lunchbox will be provided upon receipt of a lunch voucher in the exhibition catering area on Sunday and Monday at the following times:
Sunday 8 July: 13:00–14:30  Monday 9 July: 13:00–14:30

Certificate of Attendance
Following accreditation approval by ACOE, the EACR-22 Congress has been granted European endorsement by the UEMS and awarded 18 European Continuing Medical Education Credits (ECMEC). Certificates of Attendance recording CME credits will be available online immediately after the congress. You will receive an email link to a short questionnaire which also provides the link for you to print your Certificate of Attendance. We kindly ask you to keep your Congress badge as you will need the unique badge code to print your Certificate of Attendance.
The Congress Secretariat will not mail Certificates of Attendance to participants after the congress. For information on CME accreditation see page ix.

Cloakroom
A cloakroom is located on level −1, accessible by stairs in the entrance hall.
Cloakroom opening hours:
Saturday 7 July: 12:00–21:30
Sunday 8 July: 07:30–21:00
Monday 9 July: 07:30–19:30
Tuesday 10 July: 07:30–13:30
Cloakroom rates: 2 EUR per coat or item of luggage.
Congress Dinner
The EACR-22 Congress Dinner will take place at the W Barcelona from 20:30 onwards. Ticket sales were limited owing to the capacity of the venue and sold out prior to the start of the congress. Ticket holders will be asked to present their tickets upon arrival at the restaurant.

EACR General Assembly and Awards Ceremony, Sunday 8 July 19:30
The General Assembly and Awards Ceremony of the European Association for Cancer Research will be held in room 113 of the congress centre. The Awards Ceremony is followed by a reception where light refreshments will be served.

Exhibition
The EACR-22 exhibition is held in the exhibition hall on the ground floor of the congress centre. Entrance is free for registered delegates.

Exhibition opening hours:
Sunday 8 July: 09:45–17:30
Monday 9 July: 09:45–17:30
Tuesday 10 July: 09:45–12:00

For an exhibition floor plan and list and profiles of exhibitors, please see the section ‘exhibition’ (p. xxi) of this Proceedings Book.

First Aid
A first aid room is located on level −1 near the cloakroom. In case of emergency, please inform the nurse on duty via +34 697324433 and/or dial internal the extension number 257 from any internal phone in the congress centre.

Insurance
The organisers of EACR-22 do not accept liability for individual medical, travel or personal insurance. Participants are strongly recommended to obtain their own personal insurance policies. The organisers of EACR-22 accept no responsibility for loss due to theft or negligence.

Internet Wi-Fi access
General Wi-Fi access is available throughout the congress centre with the exception of the exhibition halls. To access Wi-Fi, activate the Wi-Fi network and select the network listed as EACR-22.

Internet Zone
The official EACR-22 internet zone located in the entrance hall is available free of charge during the congress. The terminals provide you with internet browsing, access to web-based mail, the congress programme and exhibitor information.

EACR-22 gratefully acknowledges the support of Elsevier as sponsor of the Internet Zone

Language and Translation
The official language of the congress is English. No simultaneous translation is provided.

Lost and Found
All enquiries should be directed to the registration helpdesk in the entrance hall. The organisers accept no responsibility for loss due to theft or negligence.

Opening Lecture and Ceremony
The Opening Lecture and Ceremony, followed by a reception, will be held in room 115 of the congress centre. Access is free for all registered participants. Please refer to the Scientific Programme for further details.

Poster Sessions
Every poster will be on display in the dedicated poster area for the entire duration of the congress and during all poster sessions. On Sunday 8 July 2012 starting at 08:00 AM, poster presenters will be allowed access to the
Exhibition Hall of the congress centre to mount their poster on the poster board displaying their assigned poster number. For assistance, please check with the EACR-22 staff onsite.

**Posters must be removed on Tuesday 10 July by 12:30 PM.** Please note that any posters remaining after this time will be removed by the organisers and cannot be reclaimed.

Presenting authors are kindly requested to be present at their poster for poster defence on:
- Sunday 8 July: 16:00–17:30 (posters with an even number)
- Monday 9 July: 16:00–17:30 (posters with an odd number)

**Public Transport**

All fully registered participants (excluding day registrations) can buy a 10 journey public transport ticket in Barcelona at a reduced rate of 4.50 EUR (regular price: 9.25 EUR). Payment can be made in cash only at the public transport desk located in the entrance hall. For your convenience, a city map has been included in your congress bag.

**Registration**

EACR-22 is open to all registered participants. Your official badge is required for admission to the congress centre and all congress events. For security reasons, participants are requested to wear their badge at all times.

**Registration opening hours:**
- Saturday 7 July: 07:00–20:00
- Sunday 8 July: 07:00–18:00
- Monday 9 July: 07:00–18:00
- Tuesday 10 July: 07:00–13:00

**Registration Package**

The full congress registration package includes:
- Access to all scientific sessions and to the Opening Lecture and Ceremony on Saturday 7 July;
- Entry to the exhibition;
- EACR-22 coffee breaks and two lunchbox vouchers;
- EACR-22 Congress bag including the EACR-22 Proceedings Book, the journal *Molecular Oncology*, the latest issue of *EJC* and a city map;
- Internet access via the internet zone and Wi-Fi access in the congress centre.

The day registration package includes:
- Access to scientific sessions on that day;
- Entry to the exhibition;
- EACR-22 Congress bag and contents depending on availability;
- EACR-22 coffee breaks on that day, and a lunchbox voucher if applicable on that day;
- Internet access via the internet zone and Wi-Fi access in the congress centre.

**Satellite Symposia**

Several Satellite Symposia are taking place during EACR-22. For schedules and more information see the section ‘Satellite Symposia’ on page xxix.

**Social Media**

Twitter is available during the congress – tweet, network, and follow updates at #EACR22.

**Speaker Preview Room**

The Speaker Preview Room is located in room 134 (first floor). Speakers are requested to take their PowerPoint presentations to the Speaker Preview Room at least 4 hours before their session starts or one day in advance if the session starts early in the morning. To ensure the smooth running of the sessions and avoid lengthy breaks in between speakers, the use of laptops in the session rooms is actively discouraged.

**Speaker Preview Room opening hours:**
- Saturday 7 July: 07:00–18:30
- Sunday 8 July: 07:00–18:00
- Monday 9 July: 07:00–18:00
- Tuesday 10 July: 07:00–12:00
17th ECCO - 38th ESMO - 32nd ESTRO
European Cancer Congress
Reinforcing multidisciplinarity
AMSTERDAM, 27 SEPTEMBER - 1 OCTOBER 2013

www.ecco-org.eu
Awards

Mike Price Gold Medal Award 2012
Award winner: Dr. José Baselga

José Baselga is the Chief of the Division of Hematology/Oncology and Associate Director of the Massachusetts General Hospital Cancer Center in Boston where he is also Professor of Medicine at Harvard Medical School. His research interests are in clinical breast cancer and in translational and early clinical research. He conducted the initial clinical trials with the monoclonal antibodies cetuximab and trastuzumab and is leading the clinical development of several new agents including pertuzumab and PI3K inhibitors. His main focus in the laboratory and in the clinic is in the area of novel anti-HER2 agents, in the identification of mechanisms of resistance to anti-HER2 agents and therapeutic approaches to target the PI3K pathway. He is also leading a number of neo-adjuvant trials in breast cancer and has been at the forefront of developing biomarker-based early and translational clinical trials.

Dr. Baselga received his M.D. and Ph.D. degree from the Universidad Autonoma of Barcelona. He completed a fellowship in Medical Oncology at Memorial Sloan-Kettering Cancer Center in New York and subsequently stayed on as a faculty member of the Breast Medicine Service at Memorial Sloan-Kettering. From 1996 to 2010 he was the Chairman of the Medical Oncology Service and Founding Director of the Vall d’Hebron Institute of Oncology (VHIO) at the Vall d’Hebron University Hospital in Barcelona, Spain.

A recipient of many awards during his distinguished career, Dr. Baselga was most recently the recipient of the Queen Sofia Spanish Institute’s Gold Medal. He was selected for this prestigious award for representing Spain’s leadership in the area of cancer research.

Dr. Baselga is a member of the Editorial Boards of Cancer Cell, Clinical Cancer Research and Annals of Oncology and he is the founding editor-in-chief on the new flagship journal Cancer Discovery.

The Pezcoller Foundation – EACR Cancer Researcher Award
‘Celebrating academic excellence and achievements in the field of cancer research’

Award Winner: Prof. Eric CW So

The Pezcoller Foundation – EACR Cancer Researcher Award Lecture will be presented for the first time at EACR-22 and at future EACR biennial congresses.

Eric CW So obtained his PhD (Pathology) in 1997 from the University of Hong Kong and received most of his postdoctoral training at Stanford University, California where he worked with Professors Michael Cleary and Irving Weissman to study the transformation mechanisms and origins of leukemic stem cells (LSC). In 2004, Prof. So joined the newly formed Hemato-Oncology Department led by Professor Mel Greaves in the Institute of Cancer Research in London to set up his laboratory studying the molecular and cellular basis of acute leukaemia with a major focus on transcriptional and epigenetic regulation in LSC. In 2009, Professor So was appointed as the Chair Professor of Leukaemia Biology in King’s College London, and established the section of Leukaemia and Stem Cell Biology. Over the past few years, his team has made many seminal discoveries that are critical to the current understanding of leukaemia biology and to design effective therapy for the disease. His works are featured in many highly respected journals in the field including Cancer Cell, Cell Stem Cell, Nature Cell Biology and many others.
Professor So serves in the review panels and advisory committees of many scientific and medical journals as well as international funding agencies in Europe, the US and Asia. He has won many personal awards throughout his scientific career. The most recent of these are an International Fellowship from the Association for International Cancer Research (AICR) in the UK (2005–2011) and the Young Investigator's Award from the European Molecular Biology Organization (EMBO) (2008–2011). He is also an elected Fellow of Academy of Life Science for Chinese in the UK (ALSC-UK) (2010) and an elected Member of European Research Institute for Integrated Cellular Pathology (ERI-ICP) (2010).

Anthony Dipple Carcinogenesis Award 2012
Award Winner: Dr. Carlo M. Croce

The Anthony Dipple Carcinogenesis Award is for major contributions to research in the field of carcinogenesis.

Dr. Croce is world-renowned for his contributions involving the genes and genetic mechanisms implicated in the pathogenesis of human cancer. A native of Milan, Italy, Dr. Croce earned his medical degree summa cum laude in 1969 from the School of Medicine, University of Rome. He began his career in the United States the following year as an associate scientist at the Wistar Institute of Biology and Anatomy in Philadelphia. In 1980, he was named Wistar Professor of Genetics at the University of Pennsylvania and Institute Professor and Associate Director of the Wistar Institute, titles he held until 1988. From 1988–91, he was director of the Fels Institute for Cancer Research and Molecular Biology at Temple University School of Medicine in Philadelphia.

In 1991, Dr. Croce was named Director of the Kimmel Cancer Center at Jefferson Medical College at the Thomas Jefferson University, in Philadelphia. While at Jefferson, he discovered the role of microRNAs in cancer pathogenesis and progression, implicating a new class of genes in cancer causation. In 2004 he moved to The Ohio State University. Under his direction at Ohio State faculty within the Human Cancer Genetics Program conduct both clinical and basic research. Basic research projects focus on how genes are activated and inactivated, how cell-growth signals are transmitted and regulated within cells, and how cells interact with the immune system. Clinical research focuses on discovering genes linked to cancer and mutations that predispose people to cancer.

Dr. Croce, a member of the National Academy of Sciences and Institute of Medicine in the US and the Accademia Nazionale delle Scienze detta dei XL in Italy, was recently elected to Membership of The American Academy of Arts and Sciences.

Carcinogenesis Young Investigator Award 2012
Award Winner: Dr. Lars Zender

The Carcinogenesis Young Investigator Award is for a recent, significant contribution to carcinogenesis research by an investigator under the age of 40.

Lars Zender, M.D., is Professor and Head of the Division of Molecular Oncology of Solid Tumors at the University Hospital Tübingen, Germany. Lars Zender’s work especially focuses on the identification of new cancer genes involved in liver cancer development. He developed clinically relevant mosaic (chimaeric) liver cancer mouse models, which allow for high throughput functional genomic analyses. Together with a limited number of other laboratories, Lars Zender’s group has the expertise to conduct stable RNA interference screens for the identification and validation of new cancer genes directly in vivo.

Another key aspect in the scientific work of Lars Zender is his work on cellular senescence. In particular the Zender laboratory is studying the senescence associated secretory phenotype and how senescent tumour cells and pre-cancerous cells are recognised and cleared by the immune system. Recent work from Lars Zender’s laboratory showed that a continuous antigen specific immune clearance of premalignant senescent hepatocytes is crucial for tumour suppression in the liver.
EACR Profile

The European Association for Cancer Research

Information about EACR and a membership invitation to non-members attending EACR-22

With over 9,500 members, the ‘European Association for Cancer Research’ is Europe's largest member society for cancer research. As an EACR-22 participant we invite you to join the Association and be part of its further development and success.

EACR

• Is at the centre of cancer research in Europe

Founded in 1968, EACR enjoys particularly strong links with other European cancer societies: the Association was not only a founding member of the Federation of European Cancer Societies (FECS) but now maintains its central position in European activities as a founding member of the European CanCer Organisation (ECCO).

• Aims to Advance Cancer Research

EACR has ‘The Advancement of Cancer Research’ as its guiding aim. The Association presents educational, training and scientific meeting opportunities, and facilitates communication, interaction and collaboration between the cancer researchers who make up its membership. EACR membership is mainly European but presently extends to 90 countries worldwide.

• Takes an inclusive approach to membership

The size and range of membership reflects the fact that EACR sets out to be an inclusive, multi-disciplinary organisation and maintains a low membership subscription to ensure that cost does not discourage participation. Postgraduate students may obtain free membership for up to four years and the annual subscription rate for active members is just 40 Euros. Many senior researchers who support postgraduate students, post-docs and those in the early years of their career recommend EACR membership as part of support for professional development.

• Works with a growing number of National Member Societies

EACR is delighted to have a strong relationship with national cancer societies and associations across Europe. Researchers who are members of EACR affiliated ‘National Societies’ in Germany (AEK), Spain (ASEICA), Belgium (BACR), Britain (BACR), Denmark (DSCF), Croatia (HDIR), Ireland (IACR), Israel (ICS), Turkey (MOKAD), Hungary (MOT), Serbia (SDIR), France (SFC) and Italy (SIC) automatically become individual members of EACR as part of the wider benefits of belonging to their national society.

• Offers opportunities for Travel Fellowships

EACR Travel Fellowships, co-funded by the ‘Association for International Cancer Research’ (AICR), are increasingly popular and the quality of applications is particularly high. Reports from fellowship winners are published in the EACR Year Book each April and can be read by visitors on the EACR website www.eacr.org. There is no doubt that working in a new context, in a centre of excellence, is both a powerful experience and provides great encouragement to researchers.

• Supports members with Meeting Bursaries and Awards

The Association also offers support and encouragement through Meeting Bursaries and Awards. Forty-four students and early-career researchers have received support through the Association's Bursary scheme to attend EACR-22.

‘Poster Awards’ are provided by EACR and the ‘Carcinogenesis Awards’ are presented by Oxford University Press. EACR’s most prestigious awards are the ‘Mike Price Gold Medal Award’ and the ‘Pezcoller Foundation – EACR Cancer Researcher Award’ which will be presented biennially at the EACR congress.
• Offers biennial meetings through the series of EACR and multidisciplinary European Cancer Congresses
As well as providing members with its biennial congress, now in its 22nd edition, EACR also makes a powerful contribution to the scientific programme of the European Cancer Congress which will be held in Amsterdam in 2013.

• Sponsors meetings and presents workshops and symposia
As a sponsoring agency, EACR supports important scientific meetings and symposia organised in Europe, publicises the meetings and ultimately carries informative follow-up reports in the annual newsletter. Every year, the Association also presents smaller focussed meetings and courses as part of a high quality Educational Programme.

• Has the European Journal of Cancer (EJC) as its journal
The Association's journal, The European Journal of Cancer (EJC), will carry a detailed report of the EACR-22 Congress. Members enjoy a special subscription rate to the online version of EJC.

• Would like you to find out everything about EACR while in Barcelona
Visit the EACR stand in the Entrance Hall to learn more about the Association and to pick up a free copy of the Association's 2012 Year Book. You can also learn more about EACR and cancer research activities in Europe by visiting www.eacr.org. The Association's website is not only an excellent source of information but also carries application forms for fellowships, bursaries and other member benefits. An informative e-News bulletin is regularly sent to members with links to the website. The bulletin carries advance notices and links ranging from those highlighting focused scientific meetings to those listing job and study opportunities.

And

• Invites non-members to join the Association with a special offer
Applications to join EACR are managed through the Association's office in Nottingham and can be made online at any time: www.eacr.org. Non-members attending EACR-22 may enjoy membership ‘free of charge’ for the remaining months of 2012 when joining in advance for the 2013 membership year.

Robert Kenney, EACR Executive Director
EACR Secretariat
School of Pharmacy
University of Nottingham
Nottingham
NG7 2RD, UK
Tel.: +44 115 951 5114
Fax: +44 115 951 5115
Email: robert.kenney@nottingham.ac.uk
In an effort to improve breast cancer care on a global scale, Komen funds researchers at several European institutions, and around the globe, who are harnessing cutting-edge technologies to better understand the heterogeneity of the many subtypes of breast cancer and add to our growing knowledge of genome-based medicine.

This effort is based on the widespread belief that genome-based medicine, frequently called personalised medicine, is the future of cancer care. In the wake of the human genome project and rapidly advancing technologies, it has become evident that breast cancer encompasses many different genetic abnormalities, which greatly affect patients’ outcomes and response to therapy. Identifying these differences is key to treating the right patient with the right therapy at the right time.

Breast cancer research is an ongoing global initiative. Susan G. Komen recognizes the growing global impact of breast cancer and the shared challenges among countries worldwide, and since 1982 has funded 125 international breast cancer research grants totalling more than $35 million. Komen currently funds 30 international grants, which total nearly $16 million in support, including 8 Komen Scholars—Komen's breast cancer think tank—and a multi-million dollar, collaborative, cross-disciplinary Promise Grant.

Two separate studies in Switzerland, led by Giuseppe Viale, a Komen Promise Grant recipient, and Nancy Hynes, a Komen Scholar, are investigating the genetic variations that make some breast cancers resistant to therapy. The goal is to identify biomarkers that predict whether a patient will respond to therapy, maximising the efficacy of treatment and avoiding unnecessary side effects.

Another project in Switzerland, led by Komen grantee Mats Lambe, is investigating the genetic differences in pregnancy-associated breast cancers and whether these differences can be used to predict the risk or prognosis of this poorly understood cancer. Knowing the various genetic characteristics of pregnancy-associated breast cancer will aid in the early detection of these tumours and the development of personalised treatments for this unique cancer.

Komen Scholar Angelo Di Leo in Italy is conducting a clinical trial in patients with basal-type breast cancer to determine whether circulating tumour cells – tumour cells present in breast cancer patients’ blood – can predict tumour subtype, patient outcome, and response to chemotherapy.

“I find breast cancer a disease where advances in biology and patient care could be part of the same aspect,” says Dr. Angelo Di Leo, a Komen Scholar from the Sandro Pitigliani Medical Oncology Unit in Italy.

“This is an exciting field because it is possible to shorten intervals elapsing between the time a new lab discovery happens and the time this discovery impacts on patient care,” adds Di Leo.

Alan Ashworth, a Komen Scholar in the UK, is using molecular profiling to identify and integrate several different types of genetic data from breast tumours, which can be used to provide novel targets for therapies and biomarkers to direct the use of these therapies.

In Belgium, Komen Scholar Christos Sotiriou is identifying the different genetic defects of invasive lobular carcinoma (ILC), the second most common type of breast cancer. The data from these studies have already resulted in a “genomic grade index signature,” which increases the accuracy of prognosis for ILC patients, compared to pathology reports alone.

The research approaches taken by these investigators represent a multifaceted approach to characterising the idiosyncrasies of the many subtypes of breast cancer. Through their efforts, these researchers are identifying more effective therapies and tools that will help us move closer to our goal: a world without breast cancer.
FROM BENCH TO BEDSIDE

From the Federation of European Biomedical Societies

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- Fast review time – first tier within 2-3 days
- A distinguished Editorial Board
- Open Access options are available for this journal

www.elsevier.com/locate/molonc
Floor Plans

Venue Floor Plan

LEVEL P0
Exhibition, Posters & Catering
Entrance Hall

LEVEL P1
Foyer

LEVEL M2
Meeting Rooms
Foyer

LEVEL P0
Exhibition Booths 1-4

LEVEL P1
Session Rooms
Congress Secretariat
Speaker Preview Room

LEVEL M2
Meeting Rooms
Exhibition Floor Plan
# List of Exhibitors

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*Exhibition booths 1−4 are located in the entrance hall.

This list reflects confirmed exhibitors as of 25 May 2012
Exhibitor Profiles

Abcam Booth C15
www.abcam.com
Abcam is a leading provider of high quality protein research tools. We have a comprehensive portfolio of highly characterised primary antibodies, secondaries, assay and ELISA kits, proteins and biochemicals. Our online catalogue includes a growing range of over 25,000 cancer research products.

Affymetrix Booth B6
www.affymetrix.com/ffpesolutions
Affymetrix®, part of The Genome Generation, provides cancer research tools for whole-genome analysis to single-gene validation using clinical samples, including FFPE. Our solutions for expression, cytogenetics and miRNA analysis provide the most comprehensive and integrated view of the genome to enable faster translation of cancer discoveries to cancer treatment.

Agilent Technologies Booth B5
www.agilent.com
Agilent Technologies is a leading supplier of life science research systems that enable scientists to study complex biological processes and disease mechanisms. By integrating multiple comprehensive analyses – genomics, transcriptomics, proteomics, and metabolomics – Agilent is your partner to help you generate a better pathway-level understanding of your biological system and be at the forefront of oncology research.

American Association for Cancer Research (AACR) Booth 2
www.AACR.org
The mission of the AACR is to prevent and cure cancer through research, education, communication, and collaboration. Through its programs and services, the AACR fosters research in cancer and related biomedical science; accelerates the dissemination of new research findings among scientists and others dedicated to the conquest of cancer; promotes science education and training; and advances the understanding of cancer etiology, prevention, diagnosis, and treatment throughout the world.

Atlas Antibodies AB Booth C22
www.atlasantibodies.com
Atlas Antibodies is a Swedish biotech company that manufactures and distributes highly characterised antibodies targeting all human proteins. The antibodies are originally developed and validated within the Human Protein Atlas (HPA) project. Characterisation data, including 700 IHC, WB and IF images per antibody, are easily accessible on the HPA portal.

BD Biosciences Booth C6
www.bdbiosciences.com/eu
BD Biosciences, a segment of Becton, Dickinson and Company, is one of the world’s leading businesses focused on bringing innovative tools to life science researchers and clinicians. Its product lines include: flow cytometers, cell imaging systems, monoclonal antibodies, research reagents, diagnostic assays, and tools to help grow tissue and cells.

BioScale Booth A11
www.bioscale.com
BioScale’s ViBE™ Workstation enables a new generation of protein analysis by providing highly sensitive and reproducible detection and quantitation of analytes in complex biological mixtures; Integrating simple sample preparation and microparticle techniques, with a novel, non-optical detection and quantitation technology.
Cancer Research UK
www.cancerresearchuk.org
Cancer Research UK is the world’s leading cancer charity dedicated to saving lives through research. We support research into all aspects of cancer through the work of more than 4,000 scientists, doctors and nurses. Visit our stand to learn more about the charity and to find out about opportunities for funding in the UK.

Cayman Chemical Company
www.caymanchem.com
Cayman Chemical specialises in the production and distribution of assay kits, high purity biochemicals, antibodies, and enzymes for drug discovery and basic research. Products for research in cancer, endocrinology, oxidative stress, epigenetics, and other areas are readily available. Cayman also offers custom biochemical synthesis and assay services.

Cell Signaling Technology
www.cellsignal.eu
Cell Signaling Technology® produces the highest quality activation-state and total protein antibodies for the analysis of signal transduction pathways. Our antibodies and related reagents are validated in-house for a range of assay applications. Technical support is provided by the same scientists who produce the antibodies and know them best.

Charles River
www.criver.com
Charles River’s core programmes − Biosecurity, Model Quality, Animal Welfare and International Genetic Standardisation − are designed to provide the most reliable supply of standardized products and services: research models of superior health and genetic status; consulting, staffing and training; diagnostic and genetic testing; genetically engineered models and services; early-stage discovery and imaging; and vaccine manufacturing support.

Complete Genomics
www.completegenomics.com
Complete Genomics is the whole human genome sequencing company that has developed and commercialized an innovative DNA sequencing service. The Complete Genomics Analysis Platform (CGA™ Platform) combines Complete Genomics’ proprietary human genome sequencing technology with advanced informatics and data management software.

DNA Diagnostics Center (DDC)
www.dnacenter.com
DDC provides DNA testing services to authenticate cell lines using STR DNA analysis as well as detection of mycoplasma contamination. Authentication of cell lines by STR DNA analysis is recommended scientifically for quality control and many Journals require cell line authentication for publication. DDC is a fully ISO 17025 accredited laboratory.

eancer
www.ecancer.org
eancer is the leading oncology channel committed to improving cancer communication and education with the goal of optimising patient care and outcomes. By using the latest technologies, eancer works closely with leading figures in oncology to inform and educate the global cancer community.

ECCO – the European CancEr Organisation
www.ecco-org.eu
The European CanCer Organisation (ECCO) represents the interests of over 50,000 oncology professionals. It drives multidisciplinarity in the field and connects the European cancer community by leveraging knowledge, promoting education and building awareness. ECCO also proactively engages with policymakers to promote the interests of cancer research, cancer patients, and all other oncocommunity members.
Essen BioScience Ltd

We specialise in high-throughput kinetic assays. Our biologically relevant models cover angiogenesis, migration, invasion, apoptosis, cytotoxicity and proliferation – all with real time kinetics. The IncuCyte is specially designed to observe and analyse events over extended periods (days, hours or weeks) by gathering images continuously and remotely without intervention.

European Association for Cancer Research (EACR)

The European Association for Cancer Research (EACR) is the largest member society for cancer research in Europe and has a membership of over 9,500. In seeking to advance cancer research, EACR supports its members through a wide range of activities, scientific meetings and other opportunities for communication and interaction.

Exiqon

Exiqon is a leading supplier of products for microRNA research. Researchers around the world in academia, the biotechnology industry and the pharmaceutical industry are using our research products to make groundbreaking discoveries about the correlation between gene activity and the development of cancer and other diseases.

Harlan Laboratories

Harlan Laboratories is a leading global provider of non-clinical contract research, research models, animal diets, and services to pharmaceutical, biotech, agrochemical and chemical industries, academic and government research organisations. Harlan offers a full line of proven performance oncology models, diets, and services to meet your specific research needs.

Health Protection Agency Culture Collections

The Health Protection Agency Culture Collections is a not-for-profit strategic business unit within the Health Protection Agency. The Culture Collections consists of four clinically oriented culture collections which includes the European Collection of Cell Cultures (ECACC). ECACC supplies authenticated cell lines, including approximately 600 human cancer cell lines, and related services.

IDEXX RADIL™

IDEXX RADIL offers a variety of diagnostic assays that detect infectious agents and/or cross-contamination in tissues, cell line and other types of biologic specimens. Assure the quality and validity of your cell cultures with IDEXX RADIL's full cadre of cell line authentication, pathogen, Mycoplasma and sterility testing services.

Illumina

At Illumina, our goal is to apply innovative technologies and revolutionary assays to the analysis of genetic variation and function. Our offering includes leading-edge solutions for:

- SNP genotyping
- DNA methylation studies
- Copy number variation
- Gene expression profiling
- Low-multiplex analysis of DNA, RNA, and protein

Integrated DNA Technologies

Integrated DNA Technologies (IDT) is a leader in custom biology for the research and diagnostic life science market and serves academic research, biotechnology, and pharmaceutical development. Products include DNA oligos, qPCR assays, and custom gene synthesis to support many applications such as DNA sequencing, SNP detection, and functional genomics.
Jackson Immunoresearch Europe  
www.jireurope.com  
First Class -Secondary Antibodies, designed for many scientific applications  
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- Western Blotting  
- Flow Cytometry  
- ELISA  
- In Situ Hybridisation  
- Enzyme Immunohistochemistry  
- STED Microscopy  
- 2-photon Microscopy  
Now including Alexa Fluor® 488, 594 and 647 nm dye conjugated antibodies.

JPT Peptide Technologies  
www.jpt.com  
JPT Peptide Technologies is the leading provider of peptide based products and services to monitor cellular and humoral immune responses. Our patented technologies are: PepStar™ – peptide microarrays for humoral immune response profiling; PepMix™ – for antigen specific stimulation of cellular immune responses, PepTrack™ – peptide libraries for clinical T-cell assays.

LGC Standards  
www.lgcstandards.com  
LGC Standards provides the most comprehensive range of reference materials, pharmaceutical impurity reference standards, biological standards and reagents from a single source. These are supplied to labs and scientists around the world working in analysis and research spanning a wide range of industrial sectors including food, environmental, pharmaceutical, clinical, forensic, life sciences, industrial and phytochemical.

LI-COR Biosciences  
www.licor.com  
LI-COR Biosciences is a leader in the design and manufacturing of integrated instrument systems for biotechnology, drug discovery, environmental research and plant biology. The company pioneered the development of infrared fluorescence labelling and detection systems for protein and in vivo imaging, quantitative Western blotting, DNA sequencing, genotyping, AFLP®. Contact:  
Email: franziska.heise@licor.com or  
Tel.: +49 6172 1717740

Life Technologies  
www.lifetechnologies.com  
Life Technologies Corporation is a global biotechnology company dedicated to improving the human condition. Our systems, consumables and services enable researchers to accelerate scientific and medical advancements that make life even better. The company manufactures both molecular diagnostic and research use only products.

Mirimus Inc.  
www.mirimus.com  
Mirimus specialises in engineering customized mouse models with reversible gene silencing capability by harnessing the power of RNA interference. Using the most advanced platforms of RNAi design, Mirimus generates unique mouse models that will serve as superior preclinical models for target discovery and toxicology research.

NOF America Corporation  
www.nofamerica.com  
We are a US subsidiary of NOF CORPORATION who supplies fine chemical products to the world. We will show ‘Lipidure-Coat Plates and Dishes’, low cell attachment plates, as a tool for making 3D tumour cells. You can prepare tumour spheroids so easily in a short time using Lipidure-Coat plates.
Novus Biologicals Booth B4

Novus Biologicals has over 180,000 guaranteed research products, including Antibodies, Proteins, Peptides, Kits, Lysates and more. All Novus Biologicals products are 100% guaranteed to work in the species and applications listed on the datasheet. Novus Biologicals Europe is based in Cambridge, UK and serves all European countries.

Oracle Health Sciences Booth A6

Oracle Health Sciences helps pharmaceutical, biotechnology, and medical device companies as well as integrated delivery networks and healthcare organisations to discover, develop, and market innovative products and services that prevent and cure disease, enhance quality of life, and meet shareholder expectations.

Oxford Optronix Booth A8

Oxford Optronix provides next-generation, continuous dissolved oxygen (pO2) monitors for in vitro and in vivo applications, automated colony/spheroid counters for adherent or non-adherent/soft agar colony forming assays and a new-generation hypoxic workstation.

Peprotech Booth B12

Since 1988 PeproTech focuses on the development and manufacturing of high-quality cytokine products for life-science research. Today PeproTech is a world leader in the production of recombinant proteins, monoclonal/polyclonal antibodies and ELISA kits. With >2,000 products manufactured in-house, PeproTech has refined innovative protocols to ensure activity, reliability and consistency.

PerkinElmer Booth AA2

With a growing emphasis on translational insight, it is more important than ever to be able to examine the molecular mechanisms of a disease along with therapeutic response within relevant in vivo models. PerkinElmer is the leading provider for in vivo imaging solutions with renowned expertise in imaging, reagents and application support that will help you bring it all together.

QIAGEN Booth BB3

QIAGEN is the leading global provider of sample and assay technologies. Sample technologies are used to isolate and process DNA, RNA, and proteins from biological samples such as blood or tissue. Assay technologies are used to make such isolated biomolecules, such as the DNA of a specific virus, visible for subsequent analysis.

Quilcore Booth B9

Quilcore focuses on bioinformatics software for biomedical research in the life science and biotech industries as well as academia. With Quilcore Omics Explorer you drastically shorten analysis time and add more creativity to your research, thanks to stunning 3D graphics, point and click user interface and state of the art statistical analysis.

R&D Systems Booth B17

R&D Systems offers a range of high quality reagents for studying all areas of cancer. These include a wide range of high performance antibodies, and the most referenced collection of bioactive proteins and immunoassays in the industry. Products include:
- Antibodies
- Proteins
- Multiplex Assays
- Immunoassays
- Stem Cell Kits
- ELISpot Assays
- Arrays
Research Diets
www.ResearchDiets.com
Research Diets, Inc. formulates and produces purified OpenSource Diets® for laboratory animals. Our nutrition scientists consult on custom diet formulations. The BioDAQ® Food and Water Intake Monitor features spill-reducing hoppers, mounts to home cage, records the time, duration, amount of each meal automatically. Data is interpreted using powerful analysis software.

Seahorse Bioscience
www.seahorsebio.com
Seahorse XF Analyzers and stress test kits enable the measurement of the two major energy pathways of cells – mitochondrial respiration and glycolysis – in a microplate in minutes, in real-time. You can measure stem cell bioenergetics, enabling new research into aging, cancer, and metabolic, cardiovascular, and neurodegenerative diseases.

SIRION BIOTECH
www.sirion-biotech.com
SIRION BIOTECH is a technology leader in the field of functional gene analysis and sophisticated cell modeling for basic research, the drug, food and cosmetic industries. Combining its proprietary viral vector platform, RNAi technology and outstanding expertise in cells, SIRION BIOTECH enables much improved target research and compound screening.

Tocris Bioscience
www.tocris.com
Tocris Bioscience provides innovative, high performance reagents for studying major areas of cancer research including:

- Autophagy
- DNA Damage
- Growth Factor Signalling
- Metastasis

Tocris’ extensive product range includes the latest exclusive research tools in addition to established biochemical standards. Visit www.tocris.com to review our latest products and to request free literature.

Toray Industries Inc.
www.3d-gene.com/en/
The Toray ‘3D-Gene’ gene expression microarray platform, by utilising unique substrate manufacturing techniques and hybridisation technology, takes analysis to new levels of sensitivity. Researchers can now access ‘difficult’ samples such as FFPE tissue with greater confidence. Applications include whole human transcriptome and micro RNA arrays.

VisualSonics
www.visualsonics.com
VisualSonics is the undisputed world leader in real-time, in vivo, micro-imaging systems, providing modalities specifically designed for preclinical cancer research. Our systems deliver outstanding image quality and resolution down to the cellular level. These tools provide fast, accurate 3D tumour sizing, biomarker analysis, and sophisticated angiogenesis/tumour perfusion quantification.

Xclinical GmbH
www.xclinical.com
XClinical GmbH, an EDC-CDM system vendor provides solutions for the electronic conduct of all types of clinical trials, post-marketing studies and registries. XClinical develops MARVIN, a CDISC ODM-certified online platform for EDC, CDM and CTM. STUDY COMPOSER provides a graphical user interface for study setup, CRF design and DVP.
Satellite Symposia

Sunday 8 July 2012

ecancer

Satellite Symposium

Translational and Tailored, but Can We Afford It? Room 113, 13:00–14:30
Chair: G. McVie (United Kingdom)
Chair: U. Ringborg (Sweden)

13:00–13:05 Welcome and Introductions
Speaker: G. McVie (United Kingdom)

13:05–13:25 Translating Cancer Research to Personalised Hospital Treatment. Can it Work?
Speaker: J. Seoane (Spain)

13:25–13:45 Salami Slicing Breast Cancer – Cheap and Cheerful?
Speaker: C. Caldas (United Kingdom)

13:45–14:05 Cost Efficiency of Targeted Therapy
Speaker: M.G. Daidone (Italy)

14:05–14:25 Society and Money: the Cancer Conundrum
Speaker: R. Sullivan (United Kingdom)

14:25–14:30 Closing Remarks
Speaker: U. Ringborg (Sweden)

illuminatm

Satellite Symposium

Cancer Genomics: Transforming our Understanding of Cancer Room 111, 13:00–14:30

Illumina’s Comprehensive Portfolio for Cancer Research
Speaker: L. Smink (United Kingdom)

High Throughput Sequencing in Cancer Genome Analysis
Speaker: I. Gut (Spain)

Towards Personalised Cancer Medicine in Norway: A Pilot Study
Speaker: L. Meza-Zepeda (Norway)

Illumina Genome Network Cancer Analysis Service
Speaker: T. Boyaniwsky (United Kingdom)
Monday 9 July 2012

**Satellite Symposium**

**Unlock the Full Potential Inside Your FFPE Samples**  
Room 111, 13:00–14:30

13:00 Affymetrix Solutions for Discovering Genomic Biomarkers from Archived Cancer Tissue  
*Speaker: J. McGregor (United Kingdom)*

13:30 Expression Profiling of Archival FFPE Samples  
*Speaker: J. Hall (United Kingdom)*

14:00 Discovering and Developing Cancer Expression Biomarkers from FFPE Tissue into Clinical Application  
*Speaker: A. Tanney (United Kingdom)*

**Satellite Symposium**

**Illuminating Pathways for Disease Research**  
Room 113, 13:00–14:30

13:00 Integrated Analyses that Use Transcriptomics, Metabolomics, and Proteomics Data  
*Speaker: B. Gordon (US)*

13:30 Highly Efficient Enrichment and Optimal Base Coverage of Small Genomic Targets Using HaloPlex Technology  
*Speaker: W. Kloosterman (The Netherlands)*

14:00 An Integrated Transcriptomic and Proteomic Profiling Approach to Determine the Pharmacodynamic Action of Sulforaphane in Human Breast Epithelial Cells  
*Speaker: A. Pandey (US)*
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<td>13:00–13:15</td>
<td>Opening Address (Room 115)</td>
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<tr>
<td></td>
<td>Welcome to EACR 22</td>
<td>Julio E. Celis, EACR President and Congress Chair</td>
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<td>Message from the National Committee Chair</td>
<td>J. Seoane (Spain)</td>
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<td>13:15–14:15</td>
<td>Mühlbock Lecture (Room 115)</td>
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<td>13:15</td>
<td>Lgr5 Stem Cells in Self-renewal and Cancer</td>
<td>Speaker: H. Clevers (The Netherlands)</td>
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<td>14:15–15:00</td>
<td>ASEICA Lecture: Deconstructing Metastasis (Room 115)</td>
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<td>Chair: C. Isacke (United Kingdom)</td>
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<td>14:15</td>
<td>Micrometastasis Niches, Signals and Pathways</td>
<td>Speaker: J. Massagué (USA)</td>
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<td>15:30–17:15</td>
<td>Symposium: Cancer Genomics (Room 115)</td>
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<td>Chair: E.R. Mardis (USA)</td>
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<td>15:30</td>
<td>Genomic Analysis of Human Tumours for Guiding Therapeutic Selection</td>
<td>Speaker: C.M. Perou (USA)</td>
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<td>16:00</td>
<td>The Genomic Landscapes of Breast Cancer and Their Clinical Relevance</td>
<td>Speaker: C. Caldas (United Kingdom)</td>
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<td>16:45</td>
<td>Using Whole Genome Sequencing for Digital Analysis of Tumour Evolution</td>
<td>Speaker: E.R. Mardis (USA)</td>
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<tr>
<td>15:30–17:15</td>
<td>Symposium: Immunotherapy of Cancer as the Most Personalised Cancer Treatment (Room 111)</td>
<td>Abstract number</td>
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<td>Chair: L. Zitvogel (France)</td>
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<tr>
<td>15:30</td>
<td>Immunotherapy of High Risk HPV Infections</td>
<td>Speaker: C.J.M. Melief (The Netherlands)</td>
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<tr>
<td>16:00</td>
<td>Immunogenicity of Cell Death for Optimal Efficacy of Chemotherapy</td>
<td>Speaker: G. Kroemer (France)</td>
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<tr>
<td>16:45</td>
<td>Novel Predictors of Response to Tyrosine Kinase Inhibitors: The Paradigm of KIT+ Malignancies and NK Cell Attack</td>
<td>Speaker: L. Zitvogel (France)</td>
</tr>
</tbody>
</table>
15:30–17:15 Symposium: DNA Damage and Genome Instability (Room 113)  
Chair: J. Bartek (Denmark)

15:30 Unexpected New Insights Into ATM and p53  
Speaker: M. Kastan (USA)  

16:00 The Molecular Basis for Replication-Induced DNA Damage in Early Stages of Cancer Development  
Speaker: B. Kerem (Israel)  

16:30 Proffered paper: The Cajal Body Protein WRAP53β – a Novel Player in the Early DNA Damage Response  
M. Farnebo, E. Hedström, M. Edgren, S. Henriksson (Sweden)  

16:45 DNA Damage and Genomic Instability in Cancer Development: Mechanisms and Opportunities for Targeted Treatment  
Speaker: J. Bartek (Denmark)  

17:15–18:00 The Pezcoller Foundation – EACR Cancer Researcher Award Lecture (Room 115)  
Chair: J. Celis (Denmark)

From Normal Cells to Cancer Stem Cells, from Transformation Mechanism to Targeted Therapy: a Leukemia Prospective  
Speaker: C.W.E. So (United Kingdom)  

19:00–21:00 Opening Ceremony: Opening Lecture and Reception (Room 115)  
Welcome Message by the EACR-22 Congress Chair  
J.E. Celis (Denmark)

Message from the Ramon Areces Foundation  
F. Mayor-Zaragoza (Spain)

Message from the Ballesteros Foundation  
Speaker to be announced  
Opening Lecture: Personalised Cancer Treatment  
Speaker: J. Mendelsohn (USA)

Message from Susan G. Komen for the Cure  
N. Brinker (USA)  

Sunday, 8 July 2012

08:00–08:50 Educational Lecture (Room 115)  
08:00 Sequencing  
Speaker: H.B. Zhang (China)  

08:00–08:50 Educational Lecture (Room 111)  
08:00 Drug Discovery and Development – a Changing Paradigm?  
Speaker: J. Yingling (USA)  

08:00–08:50 Educational Lecture (Room 113)  
08:00 How Do We Study Network Perturbations in Clinical Specimens? How do we Select “Drivers” of Malignancies?  
Speaker: S. Friend (USA)
<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
<th>Abstract Number</th>
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<tbody>
<tr>
<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 115)</strong></td>
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<tr>
<td>09:00</td>
<td>Meet the Editor – Inside Nature</td>
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<td></td>
<td>Speaker: B. Marte (United Kingdom)</td>
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<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 111)</strong></td>
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<td>09:00</td>
<td>Meet the ERC</td>
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<td>Speaker: C.H. Heldin (Sweden)</td>
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<tr>
<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 113)</strong></td>
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<td>09:00</td>
<td>The Cancer Cell Line Encyclopedia – Using Preclinical Models to Predict Anticancer Drug Sensitivity</td>
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<td></td>
<td>Speaker: J. Barretina (USA)</td>
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<tr>
<td>10:15–12:00</td>
<td><strong>Symposium: Epigenetics (Room 115)</strong></td>
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<td>10:15</td>
<td>Current Views of the Cancer Epigenome and the Translational Implications</td>
<td>23</td>
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<td></td>
<td>Speaker: S.B. Baylin (USA)</td>
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<td>10:45</td>
<td>Epigenetic Changes in Cancer: From Discovery to Deployment</td>
<td>24</td>
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<td></td>
<td>Speaker: J.G. Herman (USA)</td>
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<tr>
<td>11:15</td>
<td>Proffered Paper: Involvement of Epigenetics in the Early Stages of NSCLC Development</td>
<td>25</td>
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<td></td>
<td>C. Ambrogio, F.J. Carmona, D. Santamaria, G. Gomez, M. Lozano, S. Mainardi, P. Nieto, O. Kocher, M. Esteller, M. Barbacid (Spain)</td>
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<tr>
<td>11:30</td>
<td>Human Cancer Epigenetics</td>
<td>26</td>
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<td>Speaker: M. Esteller (Spain)</td>
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<tr>
<td>10:15–12:00</td>
<td><strong>Symposium: Viruses and Cancer (Room 111)</strong></td>
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<td>10:15</td>
<td>Hepatitis C Virus – Pathogenesis, Replication and Treatment</td>
<td>27</td>
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<td>Speaker: C. Rice (USA)</td>
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<tr>
<td>10:45</td>
<td>Epstein–Barr Virus – Pathogenesis and Immunobiology</td>
<td>28</td>
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<td>Speaker: A. Rickinson (United Kingdom)</td>
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<tr>
<td>11:15</td>
<td>Proffered Paper: Detection of Hypermutation in Human Papillomavirus DNA Sequences From Cervical Samples</td>
<td>29</td>
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<td>V. Correa Vieira, J. D. Siqueira, A. R. I. Meyrelles, G. L. Almeida, H. N. Seuanez, E. S. Machado, E. A. Soares, M. A. Soares (Brazil)</td>
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<td>11:30</td>
<td>Current and Future Vaccines to Prevent HPV-associated Cancers</td>
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<td>Speaker: D. Lowy (USA)</td>
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<tr>
<td>10:15–12:00</td>
<td><strong>Symposium: Ageing and Cancer (Room 113)</strong></td>
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<td>10:15</td>
<td>Telomeres in Cancer and Ageing</td>
<td>31</td>
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<td>Speaker: M.A. Blasco (Spain)</td>
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<tr>
<td>10:45</td>
<td>Mechanisms of Ageing and Longevity</td>
<td>32</td>
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<td>Speaker: A. Brunet (USA)</td>
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<tr>
<td>11:15</td>
<td>Proffered Paper: Age-associated Cytokine Signaling Impairs Epidermal Stem Cell Function</td>
<td>33</td>
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<td>W.M. Keyes, M.A. Storer, L. Cozzuto, G. Roma, J. Doles (Spain)</td>
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<tr>
<td>11:30</td>
<td>Ageing and Cancer: the Somatotropic Link</td>
<td>34</td>
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<td>Speaker: M. Holzenberger (France)</td>
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</tbody>
</table>
12:15–13:00  EMBO Lecture: Cancer Genomics (Room 115)
Chair: A.L. Børresen-Dale (Norway)
12:15 The Genomics of Drug Sensitivity in Cancer
Speaker: M. Stratton (United Kingdom)

13:00–14:00  Young Cancer Researcher's Workshops: How to be Effective in Applying for Fellowships (Room 115)
Coordinator: A. Hutterer (Germany)

14:30–15:15  OECI Lecture: Cancer Systems Biology (Room 115)
Chair: M. Oren (Israel)
14:30 Network and Systems Biology in Cancer
Speaker: Y. Yarden (Israel)

15:15–16:00  Anthony Dipple Carcinogenesis Award Lecture (Room 115)
Chair: C.C. Harris (USA)
15:15 Causes and Consequences of microRNA Dysregulation in Cancer
Speaker: C.M. Croce (USA)

17:30–19:15  The Susan G Komen® for the Cure Supported Symposium: Susceptibility Genes (Room 115)
Chair: B. Ponder (United Kingdom)
17:30 The COGS (Collaborative Oncological Gene-environment Study): Progress and Results
Speaker: D. Easton (United Kingdom)
18:00 The Search for Rare Variants That Contribute to Cancer Susceptibility
Speaker: F. Lesueur (France)
18:30 Proffered Paper: Dissecting the Genetic Components of Breast Cancer Transcriptomes
18:45 Clinical Applications of Genome-wide Association Study Data – Lessons from Breast and Prostate Cancer
Speaker: B. Ponder (United Kingdom)

17:30–19:15  Symposium: Inflammation and Microenvironment (Room 111)
Chair: M. Karin (USA)
17:30 Targeting Cytokine Networks in Malignancy
Speaker: F. Balkwill (United Kingdom)
18:00 Tumour Promoting Macrophages and Immune Cells
Speaker: A. Mantovani (Italy)
18:30 Proffered Paper: Osteopontin and Lactadherin Drive Pro-invasive Activation of Infiltrating Macrophages and Contribute to Glioma Progression
B. Kaminska, A. Ellert-Miklaszewska, P. Wisniewski, M. Kijewska, M. Dabrowski, K. Gabrusiewicz, M. Lipko (Poland)
18:45 Control of Tumour Progression and Metastasis by Lymphocyte-Produced Cytokines
Speaker: M. Karin (USA)

17:30–19:15  Symposium: Cell Death / Autophagy (Room 113)
Chair: S. Cory (Australia)
17:30 Role of Autophagy in Cancer Metabolism
Speaker: E.P. White (USA)
18:00 Regulation of Inflammation and Cell-Death Through Interactions of RHIM-domain Protein Kinases With Caspase-8  
*Speaker: D. Wallach (Israel)*

18:30 Proffered paper: The BH3-only Protein Bim Plays a Critical Role in Hodgkin Lymphoma Cell Death Triggered by the HDAC Inhibitor Givinostat and the Multikinase Inhibitor Sorafenib  
*S.L. Locatelli, A. Guidetti, L. Cleris, A.M. Gianni, A. Anichini, C. Carlo-Stella (Italy)*

18:45 Regulation of Apoptosis by the Bcl-2 Family  
*Speaker: S. Cory (Australia)*

### Monday, 9 July 2012

<table>
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<tr>
<th>Time</th>
<th>Event Description</th>
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<tbody>
<tr>
<td>08:00–08:50</td>
<td><strong>Educational Lecture (Room 115)</strong></td>
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<tr>
<td>08:00</td>
<td>The Rise of Bioinformatics in Data-driven Cancer Research</td>
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<td><em>Speaker: P. Bork (Germany)</em></td>
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<tr>
<td>08:00–08:50</td>
<td><strong>Educational Lecture (Room 111)</strong></td>
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<td>08:50</td>
<td>Statistical Challenges in the Development of Reliable and Clinically Meaningful Biomarkers</td>
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<td><em>Speaker: L.M. McShane (USA)</em></td>
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<td>08:00–08:50</td>
<td><strong>Educational Lecture (Room 113)</strong></td>
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<td>08:00</td>
<td>The use of Metabolomics to Discover Novel Metabolic Networks and new Targets for Cancer Treatment</td>
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<td><em>Speaker: E. Gottlieb (United Kingdom)</em></td>
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<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 115)</strong></td>
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<td>09:00</td>
<td>How to Organise Cancer Research</td>
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<td><em>Speaker: N. Jones (United Kingdom)</em></td>
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<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 111)</strong></td>
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<td>09:00</td>
<td>Functional Imaging</td>
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<td><em>Speaker: M. Schwaiger (Germany)</em></td>
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<td>09:00–09:45</td>
<td><strong>Meet the Expert (Room 113)</strong></td>
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<td>09:00</td>
<td>Food, Nutrition, Physical Activity, and Cancer Prevention – World Cancer Research Fund (WCRF)</td>
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<td><em>Speaker: M. Wiseman (United Kingdom)</em></td>
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<td>10:15–12:00</td>
<td><strong>Symposium: Mouse Models (Room 115)</strong></td>
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<td>Chair: M. Barbacid (Spain)</td>
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<tr>
<td>10:15</td>
<td>Analyzing Inflammation-induced Liver and Skin Cancers in AP-1(Fos/Jun)-dependent Mouse Models</td>
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<td><em>Speaker: E. Wagner (Spain)</em></td>
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<td>10:45</td>
<td>Studying Therapy Response and Resistance in Mouse Models of Human Breast Cancer</td>
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<td><em>Speaker: J. Jonkers (The Netherlands)</em></td>
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<tr>
<td>11:15</td>
<td>Proffered Paper: Next Generation RNAi Mouse Models for Drug Discovery and Toxicology Assessment</td>
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<td><em>P. Premsrirut, C. Fellmann, G.J. Hannon, S.W. Lowe, J. Zuber, S. Elledge (USA)</em></td>
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</table>
11:30 Validation of Therapeutic Targets in K-Ras Driven Lung and Pancreatic Tumors  
**Speaker:** M. Barbacid (Spain)

### 10:15–12:00 Symposium: Stem Cells (Room 111)  
**Chair:** A. Trumpp (Switzerland)

- **10:15** Cancer-initiating Cells in Glioblastoma  
  **Speaker:** J. Seoane (Spain)

- **10:45** Interactions Between Breast Cancer Stem Cells and their Niche Govern Metastatic Colonization of the Lung  
  **Speaker:** J. Huelsken (Switzerland)

- **11:15** Proffered Paper: Regression of Metastatic Melanoma by Targeting Melanoma Stem Cells  
  H. Abken, P. Schmidt, M. Schlaak, A.A. Hombach, C. Bangard, P. Kurschat, P. Zigrino (Germany)

- **11:30** Circulating Metastasis-initiating Cells in Breast Cancer  
  **Speaker:** A. Trumpp (Germany)

### 10:15–12:00 Symposium: Molecular Insights into Invasion and Drug Resistance (Room 113)  
**Chair:** D.A. Haber (USA)

- **10:15** Acquired Resistance to Targeted Therapies  
  **Speaker:** J.A. Engelman (USA)

- **10:45** Pancreatic Cancer Therapies  
  **Speaker:** D. Tuveson (United Kingdom)

- **11:15** Proffered Paper: PTEN Phosphorylation by Fibroblast Growth Factor Receptors and SRC Mediates Resistance to Epidermal Growth Factor Receptor Inhibitors in Glioblastoma  
  F. Furnari, T. Fenton, H. Zhou, S. Marie, P. Mischel, W. Cavenee (USA)

- **11:30** Molecular Characterization of Circulating Tumour Cells  
  **Speaker:** D.A. Haber (USA)

### 12:15–13:00 FEBS Lecture: Drug Development (Room 115)  
**Chair:** M. Yaniv (France)

- **12:15** Targeted Cancer Therapy Development  
  **Speaker:** A. Ullrich (Germany)

### 13:00–14:00 Young Cancer Researcher's Workshops: Women in Science (Room 115)  
**Coordinator:** M.A. Blasco (Spain)

### 14:30–15:15 Ramon Areces Foundation Lecture: Non Coding RNA (Room 115)  
**Chair:** R. Marais (United Kingdom)

- **14:30** The ceRNA Hypothesis and the Non-coding Revolution in Cancer Research and Therapy  
  **Speaker:** P.P. Pandolfi (USA)

### 15:15–16:00 Carcinogenesis Young Investigator's Award Lecture (Room 115)  
**Chair:** C.C. Harris (USA)

- **15:15** Immune Surveillance of Senescent Cells-biological Significance in Cancer- and Non-cancer Pathologies  
  **Speaker:** L. Zender (Germany)
17:30–19:15 Symposium: Targeted Therapies/Signalling Pathways (Room 115)  
Chair: A. Levitzki (Israel)

17:30 Specific Fluorescent Probes for Glioma Cells in Living Cells and Tissue – Tools for Guided Resection  
Speaker: D. Arndt-Jovin (Germany)  
Abstract number 71

18:00 Targeting JAK-STAT Signaling for Cancer Therapy  
Speaker: R. Jove (USA)  
Abstract number 72

18:30 Proffered Paper: Glial Cell Line-derived Neurotrophic Factor (GDNF) Signaling as a Target for Endocrine Therapy Resistance in Breast Cancer  
A. Morandi, Q. Gao, P. Francica, A. Mackay, J.S. Reis-Filho, M. Zvelebil, M. Dowsett, L.A. Martin, I. Plaza-Menacho, C.M. Isacke (United Kingdom)  
Abstract number 73

18:45 Targeting the Immune System to Cancer  
Speaker: A. Levitzki (Israel)  
Abstract number 74

17:30–19:15 Symposium: Angiogenesis (Room 111)  
Chair: K. Alitalo (Finland)

17:30 Mechanisms of Resistance to Anti-VEGF Treatment – New Therapeutic Strategies  
Speaker: A.L. Harris (United Kingdom)  
Abstract number 75

18:00 Anti-angiogenesis: Novel Strategies  
Speaker: P. Carmeliet (Belgium)  
Abstract number 76

18:30 Proffered Paper: The Pro-angiogenic Phenotype of Natural Killer Cells Infiltrating Squamous Cell Carcinoma Lung Cancer  
A. Bruno, C. Focaccetti, A. Pagani, A. Imperatori, A.R. Cantelmo, C. Capella, G. Ferlazzo, L. Mortara, A. Albini, D.M. Noonan (Italy)  
Abstract number 77

18:45 Targeting Multiple Endothelial Growth Factor Pathways  
Speaker: K. Alitalo (Finland)  
Abstract number 78

17:30–19:15 Symposium: Tumour Metabolism (Room 113)  
Chair: T.W. Mak (Canada)

17:30 Roles of p53 in the Control of Metabolic Pathways  
Speaker: K. Vousden (United Kingdom)  
Abstract number 79

18:00 Profiling Metabolism for Cancer Biomarker Discovery  
Speaker: H. Keun (United Kingdom)  
Abstract number 80

18:30 Proffered Paper: Regulation of Cellular Metabolism by Cancer Genes – Implications in Breast Cancer  
Abstract number 81

18:45 Exploiting Cancer Cell Metabolism: Can it be Done?  
Speaker: T.W. Mak (Canada)  
Abstract number 82

Tuesday, 10 July 2012

08:00–08:50 Educational Lecture (Room 115)  
Mass Spectrometric Strategies for Protein Biomarker Discovery and Validation  
Speaker: R. Aebersold (Switzerland)  
Abstract number 83
08:00–08:50 Educational Lecture (Room 111)  
Abstract number
08:00 The A-Z of Clinical Trials – Clinical Trial Design Incorporating Efficacy, Translational and Biomarker Endpoints  
Speaker: E.A. Eisenhauer (Canada)  
84

08:00–08:50 Educational Lecture (Room 113)  
Abstract number
08:00 Cancer Immunotherapy: Strategies for Enhancing its Activity in Cancer Patients  
Speaker: F. Belardelli (Italy)  
85

09:00–09:45 Meet the Expert (Room 115)  
Abstract number
09:00 Molecular Diagnostics in Breast Cancer – Getting Beyond ER, PR, HER2 and Proliferation  
Speaker: J. Reis-Filho (United Kingdom)  
86

09:00–09:45 Meet the Expert (Room 111)  
Abstract number
09:00 Biobanking  
Speaker: P.H.J. Riegman (The Netherlands)  
87

09:00–09:45 Meet the Expert (Room 113)  
Abstract number
09:00 Network and Systems Biology in Cancer  
Speaker: S. Friend (USA)  
88

10:15–12:00 FEBS Molecular OncologySupported Symposium: Personalised Medicine (Room 115)  
Abstract number
Chair: T. Tursz (France)
10:15 Interrogating the Genome to Find Mechanisms of Drug Resistance in Cancer  
Speaker: R. Bernards (The Netherlands)  
89
10:45 New Targeted Agents in Development for Cancer Treatment  
Speaker: J.C. Soria (France)  
90
11:15 Proffered Paper: Testing Individualised Treatment Strategies in Preclinical Models of Pancreatic Cancer  
91
11:30 Personalised Oncology – a Worldwide Challenge for Drug Development, Epidemiology, and Public Health  
Speaker: T. Tursz (France)  
92

10:15–12:00 Symposium: Metastases and EMT (Room 111)  
Abstract number
Chair: J. Thiery (Singapore)
10:15 MicroRNAs, EMT and Cancer Stem Cells  
Speaker: T. Brabletz (Germany)  
93
10:45 Regulation of Epithelial Plasticity and Metastasis of Breast Carcinoma by Lysyl Oxidase-like 2 (LOXL2)  
Speaker: A. Cano (Spain)  
94
11:15 Proffered Paper: EMT Inducers Catalyse Malignant Transformation of Mammary Epithelial Cells and Drive Tumorigenesis Towards Claudin-low Tumours in Transgenic Mice  
A.P. Morel, G.W. Hinkal, C. Thomas, F. Fauvet, S. Courtois-Cox, A. Wierinckx, M. Devouassoux-Shisheboran, D. Spicer, S. Ansieau, A. Puisieux (France)  
95
11:30 Identification of EMTed Carcinoma and Potential Therapeutic Strategies  
Speaker: J. Thiery (Singapore)  
96
10:15–12:00 Symposium: Melanoma, an Example of Translational Research
(Room 113)
Chair: R. Marais (United Kingdom)

10:15 The Basics of Melanoma Cell Signalling
Speaker: R. Marais (United Kingdom)

10:45 Treating Metastatic Melanoma With the Braf Inhibitor Vemurafenib and Mechanisms of Resistance
Speaker: P. Chapman (USA)

11:15 Proffered Paper: The Importance of Glycolysis for the Response and Resistance of BRAFV600E Human Melanoma Cells to Vemurafenib
T. Parmenter, G. Bollag, R. Hicks, R. Johnstone, G. McArthur (Australia)

11:30 The Immuno Side of Melanoma
Speaker: A. Ribas (USA)

12:00–12:40 Closing Ceremony: Closing Remarks (Room 115)

12:00 EACR-22 Highlights and Closing Remarks
J.E. Celis (Denmark)
J. Seoane (Spain)

12:05 Poster Awards

12:10 Message from the President of the Asociación Española Contra el Cancer and Fundación Científica
Isabel Oriol (Spain)

12:15 Message from the Director of the Vall d’Hebron Institute of Oncology
J. Tabernero (Spain)

12:20 Messages from Spanish Authorities
To be Announced

12:25 Message from SAR Princess of Asturias, President of Honour of the Asociación Española Contra el Cancer and Fundación Científica

12:35 EACR-23 Announcement
M. Oren (Israel)

12:40–13:40 Mike Price Gold Medal Award Lecture (Room 115)

12:40 The Future of Personalised Medicine
Speaker: J. Baselga (USA)

Poster Sessions (Sunday 8 July – Tuesday 10 July 2012)

Cell and Tumour Biology
Cancer Genomics, Epigenetics and Genomic Instability
Signalling Pathways
Carcinogenesis
Translational Research
Experimental/Molecular Therapeutics, Pharmacogenomics
Tumour Immunology
Radiobiology/Radiation Oncology
Molecular and Genetic Epidemiology
Prevention and Early Detection
Mühlbock Lecture: Stem Cells

Lgr5 Stem Cells in Self-renewal and Cancer

H. Clevers1, 1Hubrecht Institute, Netherlands Academy of Arts and Sciences & University Medical Centre Utrecht, Utrecht, The Netherlands

The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. We originally defined Lgr5 as a Wnt target gene, transcribed in colon cancer cells. Two knock-in alleles revealed exclusive expression of Lgr5 in cycling, columnar cells at the crypt base. Using an inducible Cre knock-in allele and the Rosa26-LacZ reporter strain, lineage tracing experiments were performed in adult mice. The Lgr5™ crypt base columnar cells (CBC) generated all epithelial lineages throughout life, implying that it represents the stem cell of the small intestine and colon. Similar observations were made in hair follicles and stomach epithelium.

Single sorted Lgr5+ stem cells can initiate ever-expanding crypt-villus organoids in 3D culture. Tracking experiments indicate that the Lgr5™ stem cell hierarchy is maintained in these organoids. We conclude that intestinal crypt-villus units are self-organizing structures, which can be built from a single stem cell in the absence of a non-epithelial cellular niche. The same technology has now been developed for the Lgr5™ stomach stem cells.

Intestinal cancer is initiated by Wnt pathway-activating mutations in genes such as APC. As in most cancers, the cell of origin has remained elusive. Deletion of APC in stem cells, but not in other crypt cells results in progressively growing neoplasia, identifying the stem cell as the cell-of-origin of adenomas. Moreover, a stem cell/progenitor cell hierarchy is maintained in early stem cell-derived adenomas, lending support to the “cancer stem cell”-concept.

Fate mapping of individual crypt stem cells using a multicolor Cre-reporter revealed that, as a population, Lgr5 stem cells persist life-long, yet crypts drift toward clonality within a period of 1–6 months. Lgr5 cell divisions occur symmetrically. The cellular dynamics are consistent with a model in which the resident stem cells double their numbers each day and stochastically adopt asymmetrically. The cellular dynamics are consistent with a model in which the resident stem cells double their numbers each day and stochastically adopt

Saturday 7 July 2012

ASEICA Lecture: Deconstructing Metastasis

Micrometastasis Niches, Signals and Pathways

J. Massagué1, 1Memorial Sloan Kettering Cancer Center, Cancer Biology & Genetics, Howard Hughes Medical Institute, New York, USA

The mechanisms that allow disseminated tumor cells (DTCs) to remain viable as tumor-initiating entities for long periods are largely unknown. An understanding of these mechanisms will help the development of treatments to eradicate DTCs and thus prevent metastasis. Recently we have identified several mechanisms that prime breast cancer and lung cancer DTCs for survival and fitness in distant organs. We found that activated Src allows DTCs in the bone marrow to robustly respond to the local survival cytokines CXCL12 and IGFl. We also found that breast cancer DTCs in the lungs support their metastasis-initiating ability by expressing tenasin-C (TNC), an extracellular matrix protein of stem cell niches. TNC enhances VEGF and NFkB signaling to support the cancer stem cell fitness of metastasis-initiating cells. The Wing path was also emerged as an enhancer of cancer cell dissemination and metastasis initiation in lung adenocarcinoma. In a fourth case, we found that the binding of tumor-associated macrophages to the leukocyte receptor LFA1-1 in DTCs triggers PI3K-Akt survival signaling by engagement of the adaptor protein Ezrin. Lastly, a CXCL1/2 paracrine loop with myeloid cells protects DTCs from the stresses of micrometastasis and chemotherapy. In preclinical models, SRC kinase inhibitors, VEGF1 blocking antibodies, and CXCL1 receptor inhibitors suppress metastasis and augment the efficacy of chemotherapy. Collectively, our evidence shows that the signals and pathways that sustain DTCs can be identified, molecularly deconstructed, and therapeutically targeted.

Saturday 7 July 2012

Symposium

Cancer Genomics

Genomic Analysis of Human Tumours for Guiding Therapeutic Selection

C. Perou1, 1University of North Carolina at Chapel Hill, Chapel Hill, USA

It is now appreciated that breast cancer is not a single disease, but instead is a spectrum of tumor subtypes with distinct cellular origins, somatic changes and somewhat predictable clinical behaviors. Prognostic factors such as stage and grade, and predictive factors such as hormone receptors and HER2 status are the main features upon which clinical decision-making has traditionally been based. Gene expression data coming from DNA microarrays, and now sequencing-based technologies, has provided additional insights into the biology of breast cancer which has resulted in the development of a number of clinically useful assays. Our work on breast tumors using genomic analyses has led to a new molecular taxonomy that identifies at least five subtypes of breast cancers (Luminal A, Luminal B, HER2-enriched, Basal-like and Claudin-low) and a normal breast-like group. Known as the “intrinsic subtypes”, these groups have revealed critical differences in incidence, survival, metastatic site specificity, and response to treatment. Importantly, the information provided by the intrinsic subtypes complements the information provided by classical clinical-pathological markers, and adds value beyond estrogen receptor (ER) status, HER2 status, and stage.

In addition to the intrinsic subtypes, many other important prognostic and predictive gene expression-based profiles have been identified including the two most widely used clinical assays that are OncotypeDX and Mammaprint. Comparative genomic analyses have shown a significant level of concordance between the intrinsic subtypes, OncotypeDX and Mammaprint; however, recent analyses have also highlighted that these tests are in fact distinct and should not be considered to be three versions of the same assay. These tests and data have lead to the identification of a subset of ER-positive patients that have an extremely good outcome, and thus, for whom adjuvant endocrine therapy alone appears sufficient. Alternatively, the remaining patients/subtypes including Luminal B, HER2-enriched and Basal-like, show significantly worse prognoses, although targeted therapies for HER2 patients have greatly improved their outcomes. Analysis of adjuvant and neoadjuvant trials also suggests that not all chemotherapeutics are equally effective on each of these high risk subtypes;
The Genomic Landscapes of Breast Cancer and Their Clinical Relevance

C. Caledas 1, 2 Department of Oncology, University of Cambridge and 1 Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Cambridge, CB2 0RE, UK

Our knowledge of the genomic architecture of breast cancers has increased exponentially over the past 6 months. The genomic and transcriptomic profiles of 2,000 breast cancers have revealed a completely novel molecular taxonomy of breast cancer with 10 clinically and biologically distinct subtypes. Sequencing of around 1,000 breast cancer exomes has characterized the extent of the mutational landscape. Deep sequencing has also unravelled the clonal heterogeneity that results from the Darwinian evolution of cancers. Further layers of molecular profiling, including miRNAs, DNA methylation and ER-ChIP-sequencing, have shown unprecedented details of the landscape. The potential for clinical application of these genomic landscapes in diagnosis, prognostication, prediction, monitoring and therapy are unparalleled and will lead to a completely new way of managing breast cancers in the clinic.

Materials and Methods:
We developed multiple bioinformatic algorithms to characterize the subclonal architecture of cancers from their whole-genome sequences, and apply these to 21 breast cancers which we have sequenced to 30- to 40-fold coverage (20 cases) or 188-fold coverage (one case).

Results and Discussion:
We find that mutational processes evolve across the lifespan of a breast tumor, with cancer-specific signatures of point mutations and chromosomal instability often emerging late but contributing extensive genetic variation. Subclonal diversification is prominent, providing insight into the dynamics of clonal expansion in breast cancer. Most point mutations are found in just a fraction of tumor cells, and often occur in several distinct clusters resulting from specific clonal expansions. We observe both large and small subclonal copy number changes, painting a picture of frequent variation in chromosomal copy number that complements the image emerging from point mutations. Every tumor studied here has a dominant subclonal lineage, representing more than 50% of tumor cells. Minimal expansion of these subclones occurs until many hundreds to thousands of mutations have accumulated, implying the existence of long-lived, quiescent lineages of cells that are capable of substantial proliferation upon acquisition of enabling genomic changes. Expansion of the dominant subclone to an appreciable mass may therefore represent the final rate-limiting step in a breast cancer's development, triggering diagnosis.

Conclusion:
The interplay of point mutations, chromosomal gains and losses and clonal expansions, leave a record of the life history of a cancer inscribed in its genome. Using whole-genome sequencing and novel bioinformatic methods, we can reconstruct this life history, painting a dynamic picture of on-going evolution and clonal expansion in breast carcinoma.

Using Whole Genome Sequencing for Digital Analysis of Tumour Evolution

E. Marais 1, 2 Washington University School of Medicine, Genetics / Molecular Microbiology, Saint Louis, USA

Next-generation sequencing approaches have revolutionized our understanding of cancer as a disease of the genome. Our group recently has developed and published methods for interpreting tumor heterogeneity by exploiting the digital nature of next-generation sequencing. I will present several examples from our work using these approaches to characterize heterogeneity changes in primary vs. metastatic disease and in pre- vs. post-treatment tumor genomes.

September 7 2012 15:30–17:15
Symposium
Immunotherapy of Cancer as the Most Personalised Cancer Treatment

C. J. M. Metief 1, 2 Departments of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands, and ISA Pharmaceuticals, Leiden, the Netherlands

Therapeutic vaccination of persistent virus infections and associated diseases including (pre-)cancer so far has largely evaded success, mainly due to the fact that insufficiently consistent and robust effector T cell responses were induced by the commonly used vaccine constructs and formulations such as recombinant viruses and bacteria, recombinant proteins, DNA constructs, Dendritic Cell (DC) vaccines and exact HLA class I binding peptides (short peptides). Problems have been severe antigenic competition from vector sequences by recombinant viruses and bacteria, insufficiently powerful T cell generation by DNA constructs, insufficient homing to lymph nodes injected DC and antigen presentation of short peptides by non-professional antigen presenting cells in vivo, causing tolerance instead of immunity. Much more robust and consistent T cell responses can be obtained by vaccination with long (28-35 amino acid long) synthetic peptides. Such immunogens are more efficiently processed and presented than intact proteins by DC and only DC can efficiently perform this task. Moreover only concentrated antigen of choice is offered and antigenic competition therefore plays no role.

In earlier work we showed that therapeutic vaccination with a synthetic long peptide (SLP®) vaccine mediated the eradication of established human papilloma virus type 16 (HPV16)-positive tumors in mice and controlled wart growth and latent virus infection in rabbits persistently infected with cottontail rabbit papilloma virus. Subsequent phase I/II studies with an HPV16 SLP® vaccine, consisting of 13 long peptides covering the HPV16 E6 and E7 antigens, in patients with advanced HPV16-positive cervical cancer, revealed that this vaccine was safe and highly immunogenic. We then tested the clinical efficacy of this HPV16 SLP® vaccine in HPV16-induced high grade vulvar intraepithelial neoplasia (VIN3), a pre-cancerous epithelial disorder, spontaneous regression of which occurs in less than 2% of patients and in which recurrence after standard treatment is high. In a phase 2 trial, 20 women with VIN3 were vaccinated three times sc in the limbs with a mix of the HPV16 E6 and E7 synthetic long peptides formulated in Montanide ISA-51. The endpoints were objective clinical responses, defined as reduction of at least 50% in lesion size (partial response) or complete regressions, and HPV16-specific T-cell responses. The vaccine was safe. At 3 and 12 months after the last vaccination an objective response was observed in 12/20 (60%) and 15/19 (79%) patients respectively. Nine of them showed a complete and durable regression of the lesions at 12 months and at 24 months. The strength of the vaccine-induced HPV16-specific T-cell response was significantly higher in the group of patients with a complete regression of their lesions compared to non-responders. Patients with large lesions were less likely to experience a complete clinical response than patients with small lesions and we ascribe this to a larger proportion of vaccine induced HPV-specific regulatory cells in the patients with large lesions.

In conclusion, treatment with the HPV16 SLP® vaccine is clearly effective in patients with established VIN disease. The SLP platform lends itself for development of therapeutic vaccines against many other chronic infections and non-viral cancers. In patients with cancer, it is attractive to combine this type of vaccination with immunogenic forms of cancer chemotherapy and with immuno-modulatory drugs.

Immunogenicity of Cell Death for Optimal Efficacy of Chemotherapy

G. Kroemer 1, 2 Institut Gustave Roussy, CNRS-UMR8125 Pavillon de Recherche 1, Villejuif, France

The supreme goal of anticancer therapy is the induction of tumor cell death. Physiological cell death, which occurs as a continuous byproduct of cellular turnover, is non-immunogenic or even tolerogenic, thereby avoiding autoimmunity. However, cancer cell death elicited by radiotherapy and some chemotherapy agents such as anthracyclines and oxalipatin can be immunogenic. Immunogenic cell death involves changes in the composition of the cell surface, as well as the release of soluble immunogenic signals that occur in a defined temporal sequence. This key then operates on a series of receptors expressed by dendritic cells (DC, the ‘lock’) to allow for the presentation of tumor antigens to T cells and for the initiation of a productive immune response. Immunogenic cell death is characterized by the early cell surface exposure of caerulinin, which determines the uptake of tumor antigens by DC. The late release of the protein high mobility group box 1 (HMG1B),
which acts on toll-like receptor 4 (TLR4), is required for the presentation of antigens from dying tumor cells. In addition, the release of ATP from dying cells causes the P2X7 purinergic receptor-dependent activation of the NLRP3 inflammasome in DC, thereby allowing them to release interleukin-1β and to polarize tumor antigen-specific CD8+ T cells towards a Tc1 cytokine pattern. We postulate that the immune system determines the long-term success of anti-cancer therapies, and that this immune response is dictated by immunogenic tumor cell death. Thus, therapeutic failure can result from failure to undergo immunogenic cell death (rather than cell death as such). Thus, agents that fail to induce immunogenic cell death cannot yield a long-term success in cancer therapy. Moreover, tumors that are intrinsically unable to undergo immunogenic cell death are incurable. Importantly, it appears that mitochondrial events determine whether cancer cells die or not in response to chemotherapy, while an endoplasmic reticulum stress (ER) response combined with autophagy determines whether this cell death is perceived as immunogenic. We suggest a series of strategies to restore the immunogenicity of cell death in the context of deficient autophagy or ER stress.


Background: Identification of tumor associated antigens that trigger T-cell responses against cancer cells remains a fundamental task for the development of successful T-cell based immunotherapies. Most methods applied so far are either indirect or restricted to selected HLA-I subtypes and represent complicated and extremely time-consuming procedures. We recently described a method which overcomes these limitations.

Material and Methods: To identify candidate tissue antigens which spontaneously cause T-cell responses the protocol combines the automated two-dimensional chromatography system PF2D to fractionate the proteome of tumor tissues with immunological testing of the resulting fractions by ELISPOT assay in an autologous setting. The protein composition of the immunogenic fractions was analyzed by peptide mass fingerprinting. Localization of candidate antigens in the corresponding tumor tissue was performed by immunohistochemical staining.

Results: When fractionating the tumor proteome of a larynx and an oropharynx carcinoma spontaneous T-cell responses against different fractions appeared. We could identify in these fractions known antigens like MUC1 or EGFR as well as novel antigens such as FABP5 and XAGE-1B. Validation of these novel antigens by overlapping polypeptides showed their potential suitability for immunotherapy. Immunohistochemical staining revealed a heterogeneous expression of these antigens in cancer tissues as well as in normal mucosa. When expanding this analysis to 10 other head and neck tumor patients using the immunogenic polypeptides identified before, significant T-cell responses were seen for all novel tumor antigens in other patients.

Conclusion: Here we successfully identified in two head and neck cancer patients several known and unknown tumor associated antigens. In an independent set of patients spontaneous T-cell responses against the same antigens could be confirmed. In future studies the clinical relevance of these antigens needs to be further substantiated. In conclusion the combined PF2D-ELISPOT method identified potential T-cell antigens which might be suitable for T-cell based immunotherapy in head and neck tumors.

[10] Novel Predictors of Response to Tyrosine Kinase Inhibitors: The Paradigm of KIT+ Malignancies and NK Cell Attack

No abstract received.

Symposium DNA Damage and Genome Instability

Saturday 7 July 2012 15:30–17:15


M. Kastan1, Y. Valentin-Vega2, J. Chen1. 1Duke Cancer Institute, Department of Pathology, Duke University, Durham NC, USA, 2St. Jude Children’s Research Hospital, Department of Oncology, Memphis TN, USA

Significant progress has been made in recent years in elucidating the molecular controls of cellular responses to DNA damage in mammalian cells. Much of our understanding of the mechanisms involved in cellular DNA damage response pathways have been directed towards susceptibility syndromes that are altered in DNA damage responses. ATM, the gene mutated in the cancer-prone, radiosensitive disorder, Ataxia-telangiectasia, is a protein kinase that is a central mediator of responses to DNA double strand breaks in cells. Once activated, ATM phosphorylates numerous substrates in the cell that modulate the cell’s response to the DNA damage. p53, one of the many targets of the ATM kinase, is a critical mediator of cell cycle changes and cell death signaling following DNA damage and other stresses. Recent new insights into roles of ATM and p53 in cellular responses to stress will be discussed, including an unexpected role for ATM in regulating mitochondrial function and modulation of cellular reactive oxygen species and a critical role for increased translation of p53 mRNA in p53 induction after DNA damage. Such mechanistic insights about these pathways provide us with opportunities to develop new approaches to target the pathways for patient benefit.

[12] The Molecular Basis for Replication-Induced DNA Damage in Early Stages of Cancer Development

B. Keram1, A.C. Bester1, E. Ozeri-Galai1. 1The Hebrew University of Jerusalem, Department of Genetics, Jerusalem, Israel

Chromosomal instability is a hallmark mark of most cancers. Recent studies have found that the instability in early stages of cancer development is caused by stress on the DNA replication, however, the molecular basis for this replication perturbation remained unknown. We have recently studied the replication dynamics in cells in which a regulator of S-phase entry and cell proliferation, the RB-E2F pathway, is aberrantly activated (Bester et al 2011). Aberrant activation of this pathway by the viral (HPV-16 E6/E7) or cellular (cyclin E) oncogenes, increases cell cycle progression through S phase and reduces the cell cycle checkpoint function. This results in accelerated replication and increased replication stress, resulting in unreplicated DNA strand breaks that are sensed by ATM and p53. Importantly, ATM and p53 responses are critical for increased translation of p53 mRNA in p53 induction after DNA damage. Increased transcription of nucleotide biosynthesis genes, mediated by expressing the transcription factor c-Myc, increased the nucleotide pool and also rescued the replication-induced DNA damage. We suggest that the low-nucleotide pool is a result of an unbalanced activation of nucleotide biosynthesis genes. Hence, we further showed that activation of the cellular nucleotide biosynthesis pathway increases the nucleotide pool and rescues the replication-induced DNA damage. Altogether, these results reveal that nucleotide insufficiency plays an important role in the replication stress and genomic instability caused by aberrant activation of the RB-E2F pathway. We have further investigated the genetic instability of this mechanism by analyzing the effect of additional oncogenes and micronutrients on the replication dynamics and genomic instability. These unpublished results will be presented and discussed.

The perturbed DNA replication in early stages of cancer development induces chromosomal instability preferentially at fragile sites. However, the molecular basis for this instability was unknown. We showed that already under normal growth conditions, replication fork progression along the fragile site, FRA16C, is slow and fork frequently stall at AT-rich sequences, leading to activation of additional (rescuing) origins. Under mild replication stress, the frequency of stalling at AT-rich sequences is further increased. Strikingly, unlike in the entire genome, in FRA16C the additional origins are not activated, suggesting that all potential origins are already activated under normal conditions (Ozeri-Galai et al., Mol Cell 2011). We further studied the replication dynamics of another fragile site FRA16D. The results of this analysis will be presented and discussed. Altogether, our results provide a mechanism explaining the replication stress sensitivity of fragile sites and thus, the basis for genomic instability during early stages of cancer.

Reference(s)


M. Farnébo1, E. Hedström1, M. Edgren1, S. Henriksson1. 1Karolinska Institutet, Oncology-Pathology, Stockholm, Sweden

Introduction: We previously identified WRAP53 as an antisense gene and regulator of the p53 tumor suppressor [1]. The encoded protein, termed WRAP53, is involved in intracellular trafficking of splicing components and the telomerase enzyme to the nuclear organelles Cajal bodies [2,3]. Knockdown of WRAP53 leads to Cajal body collapse. Germline mutations in WRAP53 cause the cancer predisposition disorder Dyskeratosis Congenita. Moreover, WRAP53 is overexpressed in cancer cell lines and high expression of WRAP53 correlates with resistance to radiotherapy in head-neck cancer patients [4]. However, the mechanism underlying the radioprotective activity of WRAP53 is still unknown.

Symposia
DNA Damage and Genomic Instability in Cancer Development: Personalised Cancer Treatment

Cheung N, Chan LC, Thompson A, Cleary ML, So CW (2007) Protein effort in designing targeted therapy (Smith et al., 2011; Zeisig et al., 2011). Development of normal and leukemic stem cells, and its implication on current protein complexes associated with CTF as potential therapeutic targets for LSC (So et al., 2003b); (3) identification of transcriptional/epigenetic components in activation of CTF (Kwok et al., 2006; Kwok et al., 2010; Kwok et al., 2009; Kwok et al., 2009) and its downstream targets, Hox. These chimeric transcription factors (CTF) play a pivotal role in converting normal cells into pre-LSC with enhanced self-renewal property, which will then acquire additional genetic and/or epigenetic events to become LSC. Understanding the development biology of LSC not only will give unique insights into the biology of the disease, but also can facilitate the development of more effective targeted therapy. In this talk, we will discuss the critical steps involved in oncogenic reprogramming of normal cells into LSC, including (1) the origin of LSC (So et al., 2003a); (2) roles of aberrant self-association as a prevalent mechanism for oncogenic activation of CTF (Kwok et al., 2006; Kwok et al., 2010; Kwok et al., 2009; So et al., 2003b); (3) identification of transcriptional/epigenetic components of protein complexes associated with CTF as potential therapeutic targets for LSC (Cheung et al., 2007; Mikesch et al., 2010; Zeisig et al., 2008; Zeisig et al., 2007); (4) identification of microRNA functions and their dysregulated targeting of the Wnt/b-catenin signalling pathway for drug resistant LMSC (Yeung et al., 2010); and (5) discovery of a novel crosstalk between Bmi-1 and Wnt/b-catenin pathways in development of normal and leukemic stem cells, and its implication on current effort in designing targeted therapy (Smith et al., 2011; Zeisig et al., 2011).

Reference(s)


Saturday 7 July 2012 17:15–18:00
The Pezcoller Foundation – EACR Cancer Researcher Award Lecture

[15] From Normal Cells to Cancer Stem Cells, from Transformation Mechanism to Targeted Therapy: A Leukemia Prospective

C.W.E. So, 1 King’s College London, Department of Haematological Medicine, SES 9NU, United Kingdom

In recent years, emerging evidence suggests that a significant number of solid tumors and haematological malignancies are organized in a hierarchy where cancer stem cells (CSC) sit at the apex to initiate and sustain the disease. These CSC are also believed to be more resistant to conventional therapies and responsible for relapse, which remains a major issue for cancer treatments. Thus targeting not only the bulk of the tumor population but also these CSC is critical to truly eradicate the disease and achieve a long-term complete remission. In acute myeloid leukemia (AML) where leukemic stem cells (LSC) have been functionally identified, the most prevalent initiating events involve chimeric fusion of the retinoic acid receptor (RARa), the core-binding factors (AML1 or CBFB), the mixed lineage leukemia protein (MLL) and its downstream targets, Hox. These chimeric transcription factors (CTF) play a pivotal role in converting normal cells into pre-LSC with enhanced self-renewal property, which will then acquire additional genetic and/or epigenetic events to become LSC. Understanding the development biology of LSC not only will give unique insights into the biology of the disease, but also can facilitate the development of more effective targeted therapy. In this talk, we will discuss the critical steps involved in oncogenic reprogramming of normal cells into LSC, including (1) the origin of LSC (So et al., 2003a); (2) roles of aberrant self-association as a prevalent mechanism for oncogenic activation of CTF (Kwok et al., 2006; Kwok et al., 2010; Kwok et al., 2009; So et al., 2003b); (3) identification of transcriptional/epigenetic components of protein complexes associated with CTF as potential therapeutic targets for LSC (Cheung et al., 2007; Mikesch et al., 2010; Zeisig et al., 2008; Zeisig et al., 2007); (4) identification of microRNA functions and their dysregulated targeting of the Wnt/b-catenin signalling pathway for drug resistant MLL LSC (Yeung et al., 2010); and (5) discovery of a novel crosstalk between Bmi-1 and MLL/Hox in development of normal and leukemic stem cells, and its implication on current effort in designing targeted therapy (Smith et al., 2011; Zeisig et al., 2011).

Reference(s)

It is a century since Paul Ehrlich hypothesized and then produced the first chemotherapeutic agent. In the 1950s, the development of insulin for diabetes, and the introduction of levodopa for Parkinson’s disease provided the first instances of personalized cancer treatment. Nowadays, such tailored approaches are firmly established in the oncology setting and are extending to other medical specialties. In this lecture, I shall outline the fundamentals of personalized treatment, and discuss how we are realizing the dream of making a cancer treatment work for each patient. I shall then present the current challenges in the implementation of personalized treatment and how we can address them.

Personalised Cancer Treatment

J. Mendelsohn1. 1University of Texas, Houston Texas, USA

It is a great honor to deliver the opening lecture at this EACR meeting, which has the theme “From Basic Research to Personalized Cancer Treatment”. Tremendous advances in cancer care have been made possible by new instruments for identifying genetic and other molecular aberrations, new analytic tools, and new forms of clinical trials for validating biomarkers and investigating targeted cancer therapies. I shall review some of these accomplishments at the meeting.

It is a century since Paul Ehrlich hypothesized and then produced the first targeted chemotherapy – in his case against an infectious agent. In the period 1975–2000 we learned that aberrant gene function causes cancer, and academic and pharmaceutical researchers immediately began to develop therapies that target the products of these genes. We also sequenced the first complete human genome. Now, just over a decade into the 21st century, our progress in discovering targeted therapies and our capacity to identify a large menu of potential biomarkers in each patient’s cancer are truly staggering.

The greatest advances have been made with instruments and analytic tools that identify mutations and other abnormalities in genes. We also are making advances in proteomics, metabolomics, lipids, and other “omics.” But three other important research areas need our increased attention if we are to achieve our shared goal of delivering personalized care that reduces, or substantially delays, deaths from cancer: (1) pharmacogenomics, and variations between patients in pharmacodynamics; (2) the biological environment of connective tissue and inflammatory cells around the patient’s tumor; and, (3) the social and behavioral environments which impact the risk of dying from cancer, e.g., smoking, diet and exercise. And there are additional challenges we need to address in the clinical setting, such as patient’s expectations and rights of privacy, increased physician and patient understanding of clinical genetics and risk, better integration of clinical data and research data, and better integration of subspecialists in delivering cancer care.

In summary personalized and predictive cancer therapy is the future, and our research must address it at many levels. During the next decade we can anticipate that new approaches to determining each patient’s optimal treatment will move from being a specialized, often academic, activity to becoming a general and widespread standard of medical practice, improving the care and the outlook for all cancer patients.

Saturday 7 July 2012 19:00–21:00
Opening Ceremony: Opening Lecture and Reception
Meet the Expert

Meet the ERC
C. Heldin1, Uppsala University, Ludwig Institute for Cancer Research, Uppsala, Sweden

The European Research Council (ERC) is a novel instrument for EU funding of excellent frontier research in Europe. The budget for ERC is 7.5 bnEUR for the period 2007–2013. ERC is led by a Scientific Council of 22 European scientists covering all scientific fields. The implementation and daily work of ERC is carried out by an Executive Agency in Brussels with more than 300 scientists working together. Here are some of the issues to be discussed:

- ERC provides an opportunity to increase the quality and competitiveness of Europeanscience.
- ERC is carried out at an Executive Agency in Brussels with more than 300 scientists covering all scientific fields. The implementation and daily work of ERC is carried out by an Executive Agency in Brussels with more than 300 scientists working together.

Meet the Editor – Inside Nature
B. Marte1, Nature Publishing Group, London, United Kingdom

In this session, participants can find out more about scientific publishing. I will discuss how the peer-review process at Nature and other Nature journals works, who the editors are and how we decide which primary research papers are published in Nature. As the editor at Nature involved in handling submissions in the area of cancer, I will highlight our interest in all aspects of the field, including in translational cancer research.

Meet the ERC
C. Heldin1, Uppsala University, Ludwig Institute for Cancer Research, Uppsala, Sweden

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diversity of human cancers and for which detailed genetic and pharmacological annotation is available.

Here we describe the Cancer Cell Line Encyclopedia (CCLE), a comprehensive resource of human cancer models for basic and translational research that encompasses gene expression, chromosomal copy number and massively parallel sequencing data from 947 human cancer cell lines spanning many tumor types. When coupled with pharmacological profiles for 24 anticancer drugs across 479 of the cell lines, this collection allowed identification of genetic, lineage, and gene-expression-based predictors of drug sensitivity through systematic integration of the genomic and pharmacologic datasets. In addition to rediscovering molecular features known to predict response to several drugs, we uncovered novel potential biomarkers of sensitivity and resistance to both targeted agents and chemotherapy drugs. For instance, our analysis revealed new in vitro markers associated with sensitivity to MEK inhibitors in NRAS-mutant cell lines. Also, we found that response to topoisomerase 1 inhibitors seems to be predicted largely by expression of a single gene. Finally, we observed that tissue lineage is a key predictor for sensitivity to certain compounds, providing rationale for clinical trials of these drugs in particular cancer types.

Together, our results indicate that large, annotated cell-line collections may help to identify biomarkers that allow the stratification of patients for appropriate drug treatment at the preclinical stage. The generation of genetic predictions of drug response in the preclinical setting and their incorporation into cancer clinical trial design could speed the emergence of ‘personalized’ therapeutic regimens.

Sunday 8 July 2012 10:15–12:00
Symposium
Epigenetics

[23] Current Views of the Cancer Epigenome and the Translational Implications
J. Herman1, Johns Hopkins University School of Medicine, Baltimore Maryland, USA

Epigenetic changes represent common and functionally important alterations that contribute to carcinogenesis. The ability to assess changes, particularly in DNA methylation, at the genome level provides both a comprehensive look at these alterations as well as hundreds of potential tumor specific alterations which could be used for the detection of cancer or a determinant of outcome. This talk will focus on the discovery of alterations in DNA methylation in cancer development and of such discoveries towards early detection biomarkers using sensitive molecular approaches, as well as the role specific alterations of DNA methylation play as prognostic and/or predictive biomarkers.

[24] Proffered Paper: Involvement of Epigenetics in the Early Stages of NSCLC Development
C. Ambrogio1, F.J. Carmona1, D. Santamaria1, G. Gomez2, M. Lozano3, S. Mainardi4, P. Nieto5, O. Kocher6, M. Esteller7, M. Barbacid1

Introduction: K-RAS oncogenes are involved in about 30% of Non Small Cell Lung Cancer (NSCLC), one of the tumors with worst prognosis. We undertook these studies to identify the nature of the cancer initiating cells in these tumors as well as the underlying mechanisms that drive the earliest steps of tumor development.

Materials and Methods: We developed a genetically modified mouse model of NSCLC driven by the controlled expression of a resident K-Ras

[25] Proffered Paper: Involvement of Epigenetics in the Early Stages of NSCLC Development
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Materials and Methods: We developed a genetically modified mouse model of NSCLC driven by the controlled expression of a resident K-Ras

Results and Discussion: Induction of K-Ras expression takes place in all known lung cell types. However, only SPC+ alveolar type II cells progress to form small hyperplastic clusters. Surprisingly, these areas, in spite of being morphologically indistinguishable, display two distinct expression profiles designated as T1 and T2. Whereas the T1 signature has significant similarities to that of normal areas not expressing K-Ras, the T2 signature correlates with the gene expression profiling of aggressive NSCLCs of both mouse and human origin (Sweet-Cordero et al., Nat. Genetics 2005). Detailed analysis of these signatures has revealed a subset of genes expressed in T1 lesions that are silenced in K-Ras clusters with a T2 signature. Indeed, Dnmt1 along with various Ras epigenetic silencing elements are up-regulated in T2 lesions, suggesting a putative epigenetic contribution during the initial stages of tumor development. Bisulfite sequencing analysis has revealed that the promoters of several genes expressed in the T1 hyperplastic regions and downregulated in the T2 lesions such as SerpinB5, Runx11 and Necdin are hypermethylated, suggesting widespread epigenetic inactivation in tumor initiating cells.

Conclusions:
1. Only a limited subset of K-Ras expressing SPC+ lung cells progress to yield hyperplastic areas, some of which eventually develop into adenomas and adenocarcinomas.
2. Morphologically indistinguishable hyperplastic areas display two distinct expression profiles (T1 and T2 signatures).
3. Whereas the T1 signature is closer to normal lung epithelial cells, the T2 signature overlaps with that of aggressive NSCLCs. Thus, tumor aggressiveness might be programmed during the early stages of clonal expansion.
4. The T1 and T2 signatures are characterized by different methylation patterns suggesting that epigenetics could play a role in determining the aggressiveness of KRas lesions during early stages of tumor development.
5. The tumor suppressor SerpinB5 is hypermethylated in the T2 population.

Sunday 8 July 2012 10:15–12:00
Symposium
Viruses and Cancer

[27] Hepatitis C Virus – Pathogenesis, Replication and Treatment
C. Rice1, The Rockefeller University, New York, USA

An estimated 200 million people have been infected with hepatitis C virus (HCV) with a majority unable to clear the virus. Chronic HCV infection can lead to cirrhosis, hepatocellular carcinoma, and end-stage liver disease. It is generally believed that disease results at least in part from immune mediated inflammation. Since HCV’s discovery in 1989 significant progress has been made in establishing experimental systems and unraveling the details of the virus lifecycle. Examples include infectious molecular clones, RNA replicons and a robust cell culture system based on an HCV isolate from a rare case of acute fulminant hepatitis. Definition of the human factors required for HCV entry and blunting innate immune response pathways has led to the development of a mouse model that supports HCV entry, replication and virus production. Together these tools have increased our understanding of the HCV lifecycle and revealed multiple steps for therapeutic intervention. In 2011, two direct acting antivirals targeting the HCV serine protease were approved in combination with the previous standard of care, pegylated type I interferon and ribavirin. Even for the most difficult to treat HCV genotype (genotype 1),
Epstein–Barr Virus – Pathogenesis and Immunobiology

A. Ricke1, 1Institute of Cancer Studies, University Edgbaston, Birmingham, United Kingdom

Epstein–Barr virus (EBV), a B-lymphotropic herpesvirus widespread in human populations, is aetologically linked to a range of malignancies. These include (i) three tumours of B cell origin, post-transplant lymphoproliferative disease (PTLD), endemic Burkitt lymphoma (BL) and a subset of Hodgkin lymphomas, (ii) a specific subset of T or NK cell lymphomas, often of nasal type, and (iii) nasopharyngeal carcinoma (NPC) and a minor subset of gastric carcinomas with similar histology. Each of these tumours carry episcopal EBV genomes in every malignant cell but display distinct patterns of virus gene expression. These patterns not only reflect the different contributions EBV makes to disease pathogenesis in different tumour contexts, but also have implications for immunotherapeutic targets, by restricting the number of viral antigens that are available for targeting.

To illustrate the versatility of EBV as a tumour virus, recent studies of B cell infections in vitro have shed new light on how virus-driven growth transformation, with full latent gene expression, can contribute to PTLD pathogenesis. By contrast, two more-restricted forms of latency are found in endemic BL, both of which act not to promote growth but to protect tumour cells from the pro-apoptotic effects of c-myc gene deregulation. While the c-myc transactivation is a characteristic of both endemic (EBV-positive) and sporadic (largely EBV-negative) BL, recent work is identifying additional cell gene mutations whose patterns differ between the endemic and sporadic diseases, again reflecting their subtly different pathogenetic routes. Much still remains to be learned about the biology of EBV infection in vivo, its persistence within the B cell system, its occasional transmission to T and NK cells, and its varied contributions to human lymphomagenesis.

Proffered Paper: Detection of Hypermutation in Human Papillomavirus DNA Sequences From Cervical Samples

V. Correa Vieira1, J.D. Siqueira1, A.R.I. Meyrelles2, G.L. Almeida2, M.H. Seuanez3, E.S. Machado4, A.E. Soares1, M.A. Soares1, 1Instituto Nacional de Cáncer José Alencar Gomes de Silva – INCa, Programa de Genética, Rio de Janeiro – RJ, Brazil, 2Universidade Federal do Rio de Janeiro, Hospital Universitário Clementino Fraga Filho, Rio de Janeiro – RJ, Brazil

Persistent infection with high-risk human papillomavirus (HPV) is the main risk factor for development of cervical neoplasia. However, the factors leading to viral clearance or persistence are poorly understood. It has been shown that APOBEC3 proteins have antiviral effect against several viruses, such as HIV, inducing extensive G-to-A hypermutation into the viral genome. However, its role in the HPV infection and cancer development is unknown. Recently, evidence was shown for editing of HPV-16 DNA by APOBEC3 proteins in precancerous lesions. The aim of this study was to verify the presence of G-to-A mutations introduced by APOBEC3 into HPV sequences and to investigate its correlation with HPV-associated cervical lesions. DNA was extracted from genital scrapes using a commercial kit (QIAamp DNA Mini Kit®). To detect hypermutation, a 370 bp fragment of the HPV LCR region was amplified by 3D-PCR, which enriches the amplification of AT-rich sequences. PCR products were cloned, and at least ten clones from each sample were analyzed by colony PCR. Results were sequenced and hypermutation analysis was performed using Hypermut 2.0. A total of 68 viral sequences recovered from 5 patients were analyzed. At the time of sample collection, patients had normal cytology (n=2), LSIL (n=1) or HSIL (n=2), and all were infected by HPV-16. Among the sequences analyzed, four drastically hypermutated sequences were found (p<0.05), in addition to several sequences with elevated levels of G-to-A changes. Hypermutated sequences had a total of 65 G-to-A changes versus 4 non G-to-A changes. The preferential dinucleotide context of changes was GG (58%), suggesting a predominant activity of specific APOBEC3 proteins. All altered sequences belonged to a single patient, who had normal cytology at time of sample collection and progressed to LSIL within 6 months of follow-up. These results corroborate a previous report showing evidence for APOBEC3 editing of HPV genome in vivo. However, this is the first time that HPV hypermutation is found in cervical normal samples, the absence of cytosine abnormalities, suggesting an early role of APOBEC3 proteins in the natural course of HPV cervical infection, despite clinical evolution to intraepithelial lesion. Considering that targeting APOBEC enzymes represents a potential antiviral strategy, further studies should be conducted to elucidate the role of hypermutation in the progression of HPV-associated cervical lesions.

Symposium Ageing and Cancer

Sun 8 July 2012 10:15–12:00

Telomeres in Cancer and Ageing

M.A. Blasco1, 1CNIO – Spanish National Cancer Research Centre, Molecular Oncology Programme, Madrid, Spain

Telomeres protect the ends of chromosomes against DNA repair and degradation activities. Telomere length and integrity of telomere binding proteins are both important factors in telomere protection. Additionally, telomeres are transcribed and the telomeric RNA remains associated to telomeric chromatin. Our group has demonstrated that telomere shortening below critically short telomere lengths suffices to trigger organisinal ageing and that if we stop this process of telomere shortening through reactivation of the telomerase enzyme, we are then able of both delaying ageing and increasing longevity.
Aging, long thought to be solely the byproduct of wear and tear, is actually a highly controlled process, regulated by a combination of genetic and environmental factors. Our overarching goals are: (1) to understand the molecular mechanisms by which known longevity genes regulate aging in mammals; and (2) to discover novel genes and processes that control lifespan using two genetic models for aging: the nematode C. elegans and the extremely short-lived African fish N. furzeri. We are particularly interested in the aging of the nervous system. My presentation will be focused on the importance of stem cells in the brain during aging in mammals. In the nervous system, neural stem cells (NSCs) are thought to be important for learning, memory, and mood regulation. During aging, both the pool of NSCs and their ability to give rise to new neurons decline, raising the possibility that NSC depletion may underlie part of the cognitive dysfunctions during aging. However, the molecular mechanisms that regulate the maintenance of the NSC pool throughout lifespan are largely unknown. We have found that FoxO3, a member of a transcription factor family known to extend lifespan in invertebrates, maintains the NSC pool in adult mice. Analysis of the program of genes regulated by FoxO3 in NSCs suggests that FoxO3 maintains the NSC pool in adult organisms might have important implications for counteracting brain aging in long-lived species, including humans.

Sun 8 July 2012 12:15–13:00

EMBO Lecture: Cancer Genomics

A. Brunet1, 1Stanford Center on Longevity, Medicine Genetics, Stanford (CA), USA

Ageing, long thought to be solely the byproduct of wear and tear, is actually a highly controlled process, regulated by a combination of genetic and environmental factors. Our overarching goals are: (1) to understand the molecular mechanisms by which known longevity genes regulate aging in mammals; and (2) to discover novel genes and processes that control lifespan using two genetic models for aging: the nematode C. elegans and the extremely short-lived African fish N. furzeri. We are particularly interested in the aging of the nervous system. My presentation will be focused on the importance of stem cells in the brain during aging in mammals. In the nervous system, neural stem cells (NSCs) are thought to be important for learning, memory, and mood regulation. During aging, both the pool of NSCs and their ability to give rise to new neurons decline, raising the possibility that NSC depletion may underlie part of the cognitive dysfunctions during aging. However, the molecular mechanisms that regulate the maintenance of the NSC pool throughout lifespan are largely unknown. We have found that FoxO3, a member of a transcription factor family known to extend lifespan in invertebrates, maintains the NSC pool in adult mice. Analysis of the program of genes regulated by FoxO3 in NSCs suggests that FoxO3 maintains the adult NSC pool by inducing a program of genes that preserves cellular quiescence and regulates oxygen metabolism. Because NSCs are thought to be important for cognitive function and mood regulation, the ability of FoxO3 to quiescence and regulates oxygen metabolism. Because NSCs are thought to be important for cognitive function and mood regulation, the ability of FoxO3 to

Sun 8 July 2012 13:00–14:30

OECD Lecture: Cancer Systems Biology

Y. Yanai1, 1Weizmann Institute, Biological Regulation, Rehovot, Israel

Biological systems integrate metabolic, energy and signaling networks, by maintaining dense webs of control circuits and multiple adaptors common to two or more interfacing networks. My lecture will concentrate on systemic effects in signaling networks involved in malignant cell proliferation and migration. As a starting point, I will argue that primordial signaling pathways have been replaced in the course of metazoan evolution by layered signaling networks. The driving process behind this transformation has been whole genome duplications, which established modularity, an essential feature of biological robustness. Other features of robustness include redundancy and systems control, primarily feedback and feed-forward loops that maintain homeostasis and determine the outcome of network activation. Unlike linear pathways, networks can be trained to overcome perturbations, and their control wirings are much more sophisticated. These transitions are relevant to pharmacological attempts to intercept signaling networks, as well as to the excessive reliance of oncogenic networks on 1–2 essential hubs (‘oncogene addiction’). Using the epidermal growth factor receptor (EGFR) and its kin, a kinase called HER2/ERBB2, I will exemplify defects in system control and feedback regulation, and highlight some of the currently approved drugs that target the EGFR/HER2 axis. Along with illuminating the rationale of combination therapies, the lecture will focus on acquired (secondary) resistance to molecular targeted therapies, as an example of the remarkable adaptation capacity of signaling networks.
As miRNAs have multiple targets, their function in tumorigenesis could be due to their regulation of a few specific targets, possibly even one, or many targets. A future challenge will be to identify all of the targets of the miRNAs involved in cancer and establish their contribution to malignant transformation. An additional challenge will be the identification of all of the miRNAs that are dysregulated by pathways that are consistently dysregulated in various types of human cancers. This point is of particular importance, as instead of focusing on specific alterations in protein-coding oncogenes or tumour suppressor genes – which may be difficult to treat – we could focus on their downstream miRNA targets. If these miRNA targets are crucial for the expression of the malignant phenotype and the cancer cells depend on their dysregulation for proliferation and survival, we can expect that the use of miRNAs or anti-miRNAs will result in tumour regression. Genomic analyses for alteration in miRNA genes or for copy number alterations in various human tumours by deep sequencing is in progress but has not been completed. These studies could provide additional information concerning the involvements of miRNAs in cancer and in many other diseases.

Over the past few years, we have observed a shift from conventional chemotherapy to targeted therapies, and miRNAs and anti-miRNAs will contribute extensively to the latter.

**Sunday 8 July 2012 17:30 – 19:15**

**Susan G Komen® for the Cure Supported Symposium**

**Susceptibility Genes**

**The COGS (Collaborative Oncological Gene-environment Study): Progress and Results**

D.K. Easton,® on behalf of the COGS collaboration and Breast Cancer Association Consortium.

**Strangeways Research Laboratory, Cancer Research Campaign Genetic Epidemiology Unit, Cambridge, United Kingdom**

Susceptibility to common cancers is largely polygenic, resulting from the combined effects of many genetic loci. Importantly, in genotyping technology, many common variants have been identified linking susceptibility to cancer to be identified, but these still explain a minority of the heritability. COGS is a collaborative EU funded project to characterise susceptibility in three hormone related cancers: breast, ovary and prostate. A major component of this project is a large scale genotyping effort involving more than 200,000 samples, genotyped for more than 200,000 genetic variants using a custom array (COGS). For breast cancer, more than 30 novel susceptibility variants have been identified through this project, so that more than 60 susceptibility loci have now been found. These loci together explain approximately 16% of the familial risk of breast cancer. While the risks conferred by any individual locus are small, these common susceptibility loci combine together multiplicatively, and so can be used as a genetic risk profile. Under this model, the top 10% of the population have a risk that is 2.2 fold higher than the population average, while the top 1% have a risk that is 3.6 fold. Many of the loci confer risks that are specific to the tumour pathology: in particular many loci confer risks of predominantly ER-positive or ER-negative disease. These results can be utilised (in combination with other risk factors such as reproductive history and breast density) to stratify women for targeted screening (for example, varying the age of mammographic screening, or utilising MRI), or for chemoprevention.

**The Search for Rare Variants That Contribute to Cancer Susceptibility**


1International Agency for Research on Cancer, Genetic Cancer Susceptibility, Lyon, France, 2INSERM U095, University of Nice Sophia-Antipolis, Nice, France, 3Genetic Epidemiology Laboratory, University of Melbourne, Melbourne, Australia, 4INSERM U946, Centre d’Etude du Polymorphisme Humain, Paris, France, 5INSERM U946, Institut de Cancérologie Gustave Roussy, Villejuif, France, 6Department of Dermatology, University of Utah School of Medicine, Salt Lake City, Utah, 7Cancer and Epidemiology Unit, University of Utah School of Medicine, Salt Lake City, USA

**Introduction:** Most cancers are known to cluster in families and to be influenced by several genetic and environmental factors. Known loci explain only relatively small fractions of the total genetic variance and additional strategies beyond that of traditional linkage and genome-wide association studies (GWAS) are required to identify novel cancer genes that confer substantial increased disease risk (>2-fold). As with high penetration mutations in genes like BRCA1, the IGF1R gene and CDH1, a subset of coding variants conferring disease risks are probably functionally deleterious (e.g. nonsense, splice site, frameshift variants). The very nature of such alterations suggests that genes are likely to contain several, very rare (or private) mutations, as shown from limited candidate gene studies (e.g. ATM, CHEK2, PTEN) for breast cancer studies.

**Material and Method:** Rare, moderate-risk susceptibility alleles are identifiable through sequencing of candidate genes selected on the basis of biological plausibility, or through exome sequencing in large sample sizes with cases enriched for genetic factors (family history, age, multiple primary cancers).

**Results and Discussion:** Through target sequencing we identified a rare missense substitution in MITF that was significantly more frequent in high-risk patients diagnosed with melanoma, renal cell carcinoma or both cancers, than in the general population. This mutation severely impairs SUMOylation of the transcription factor, leading to a gain-of-function role in tumorigenesis. In the context of breast cancer, exome sequencing first suggested a role for the DNA repair gene XRCC2 with a protein-truncating in one, and a likely deleterious missense variant in another multiple-case family. Additional evolutionary unlikely variants were further identified in 10 high-risk families. We performed a population-based case-control mutation screening study and confirmed that rare XRCC2 variants confer increased risk of breast cancer. Hence, our findings increase the proportion of breast cancers that are associated with homologous recombination DNA repair dysfunction and Fanconi Anemia.

**Conclusion:** Due to the rarity and to the incomplete penetrance of MITF and XRCC2 mutations, the two genes were likely to be missed by linkage or GWAS. However, the significant disease risks associated with such breast cancer susceptibility genes, combined with the development of new sequencing technologies that are being developed, such that their identification could rapidly be translated into meaningful clinical benefit, through population driven risk prediction and disease prevention strategies.

**Proffered Paper: Dissecting the Genetic Components of Breast Cancer Susceptibilities**

S. Nord®, O.C. Lingjærde®, D. Nebdal®, W. Sun®, G.G. Alnés®, P. Van Loo®, B. Naume®, A.L. Barresen-Dale®%, V.N. Kristensen®, Genetics Department, Institute for Cancer Research, OUS, Department of Informatics, UiO, Oslo, Norway, 2Department of Bioanalytics, UNC, NC, USA, 3Wellcome Trust Sanger Institute, Cambridge, United Kingdom, 4Oncology, OUS, Oslo, Norway, 5Institute for Clinical Medicine, Faculty of Medicine, UiO, Department of Clinical Molecular Biology and Lab Science, Division of Medicine, Akerhus University Hospital and UiO, Norway

**Introduction:** Breast Cancer is a complex disease, where gene expression has been proposed as a basis for tumour classification and related to clinical outcome. Our previous results suggests that SNPs in the recently discovered susceptibility genes may exert their effect through the expression of their genes in tumours, giving rise to the various subtypes. Known loci explain only relatively small fractions of the total genetic variance and additional strategies beyond that of traditional linkage and genome-wide association studies (GWAS) are required to identify novel cancer genes that confer substantial increased disease risk (>2-fold). As with high penetration mutations in genes like BRCA1, the IGF1R gene and CDH1, a subset of coding variants conferring disease risks are probably functionally deleterious (e.g. nonsense, splice site, frameshift variants). The very nature of such alterations suggests that genes are likely to contain several, very rare (or private) mutations, as shown from limited candidate gene studies (e.g. ATM, CHEK2, PTEN) for breast cancer studies.

**Material and Methods:** We dissected the influence of germline and somatic variation on mRNA levels in 92 breast carcinomas using genome wide Illumina SNP arrays genotyped on both blood and tumor DNA, and Agilent expression of tumors. Total copy number and allele specific aberrations were called using the ASCAT algorithm, adjusting for both tumor percentage and ploidy.

**Results and Discussion:** We detected 4,346 unique genes whose expression associated in cis with genetic variation, either germline or somatic, with a subset clearly correlated with the expression of 8.8% of these 4,346 genes, and somatic copy number explained 92.9% of the expression variation. A novel finding was that 5.6% of the variation in expression was allele specific with respect to copy number. The existence of selective amplification of the germline allelic variant associated with elevated expression in breast carcinomas prompted us to investigate the tumor genome for novel, potential oncogenes, and to strengthen the candidacy of others. While eQTLs target by germline and somatic variation showed limited overlap, our interrogation of the tumor genome revealed allelic specific amplification in the major, focal amplifications of the variant allele associated with elevated expression. This pattern of natural selection confirmed well known oncogenes such as ErbB2 and MDM2, and strengthening the candidacy of others including BAG4, BRIP1, TAOK1 and IGF1R.

**Conclusion:** Taken together these results suggest that the germline background plays a significant role in the expression phenotype of tumors, and influencing the genome for thousands of variants may be an effective way to elucidate the underlying genetic factors of susceptibility of complex phenotypes such as breast cancer.

**Clinical Applications of Genome-wide Association Study Data – Lessons from Breast and Prostate Cancer**

B. Ponder®, P. Pharaoh®, N. Pashayan®, 1Cancer Research UK Cambridge Research Institute, Li Ka Shing Centre, Cambridge, United Kingdom, 2University of Cambridge, Dept Oncology and Public Health & Primary Care, Cambridge, United Kingdom, 3University College London, Epidemiology and Public Health, London, United Kingdom

Polygenic inheritance of cancer susceptibility implies a distribution of risk across the population. Using the observed familial clustering of breast cancer
as a measure of the genetic component of risk, modelling suggests a wide range of risks, for example that half of all breast cancers may occur in the 12% of women at the top end of the distribution. At present, however, the identified genetic variants can explain only about 10% (breast) to 30% (prostate) of the estimated variance, and predictive power is therefore quite poor. Risk information can be used by individuals, or applied to populations. It will discuss the potential applications of current markers, and those that might be observed in future. It is likely that current approaches will never succeed in identifying more than a minority of the genetic variation that underlies risk, and in quantifying the interactions of individual genetic variants with each other, or with the environment. I will speculate on possible ways round this problem.

Sunday 8 July 2012

17:30–19:15

Symposium

Inflammation and Microenvironment

[42] Targeting Cytokine Networks in Malignancy

F. Balkwill1, Barts and The London School of Medicine and Dentistry, London, United Kingdom

Complex networks of inflammatory cytokines and chemokines are found in most experimental and human cancers and constitutive production of these mediators is a characteristic of many malignant cell lines. However, the in vitro and in vivo interdependence of these cytokines, and their significance to the human tumor microenvironment, are both poorly understood. We are studying this in two human peripheral (ovarian) cancers – high-grade serous, HGSC, and clear cell carcinoma.

Three key cytokine/chemokine mediators of cancer-related inflammation, TNF, CXCL12 and IL6, are involved in an autocrine cytokine network, the ‘TNF network’ in the malignant cells. This network has paracrine actions on angiogenesis, infiltration of myeloid cells and NOTCH signalling in both murine xenografts and human ovarian tumor biopsies. Neutrophilic antibodies or siRNA to individual members of this TNF network reduced angiogenesis, myeloid cell infiltration and experimental peritoneal ovarian tumor growth.

The dependency of network genes on TNF was demonstrated by their down regulation in tumor cells from patients with advanced ovarian cancer following the infusion of anti-TNF antibodies. Our findings define a network of inflammatory cytokine interactions that are crucial to tumor growth and validate this network as a key therapeutic target in ovarian cancer.

To translate these observations to clinical practice, we focused on IL6. IL6 is a major mediator of cancer-related inflammation in several cancers and promotes tumor growth and angiogenesis as well as evasion of apoptosis. We investigated the therapeutic activity of NT0328 (siltuximab), an anti-human IL6 antibody, in pre-clinical and clinical experiments.

There was evidence for clinical activity of the anti-IL6 antibody, with one patient of eighteen treated demonstrating a partial response and seven showing disease stabilisation for up to nine months. In terms of mechanism of action, the clinical, pre-clinical and in silico experiments showed that antibodies to IL6 can have multiple actions within the tumor microenvironment in ovarian cancer including reductions in cytokine production, tumor angiogenesis and tumor macrophage infiltrate.

We have now used a systems biology approach, combining data from phospho-proteomic mass spectrometry and gene expression array analysis, to define the best therapeutic targets within the TNF network network and to identify drugs that may synergise with cytokine and chemokine inhibitors.

Targeting autocrine tumor-promoting networks with agents such as anti-cytokine antibodies has potential to synergize with chemotherapy, other targeted treatments and immunotherapies.

[43] Tumour Promoting Macrophages and Immune Cells

A. Mantovani1, 1Istituto Clinico Humanitas, University of Milan, Rozzano (Milan), Italy

Macrophages are key orchestrators of chronic inflammation. They respond to microenvironmental signals with polarized genetic and functional programmes. M1 macrophages which are classically activated by microbial products and interferon-gamma are potent effector cells which kill microorganisms and tumor cells, induce infammation and adaptive immunity, promote cell proliferation by producing growth factors and products of the arginase pathway (ornithine and polyamines); scavange debris by expressing scavenger receptors; promote angiogenesis, tissue remodeling and repair. M1 and M2 cells represent simplified extremes of a continuum of functional states. Available information suggests that TAM are a prototypic M2 population. M2 polarization of phagocytes sets these cells in a tissue remodeling and repair mode and they secrete and smouldering and adapted chronic inflammation associated to established neoplasia. Recent studies have begun to address the central issue of the relationship between genetic events causing cancer and activation of protumour, smouldering, non resolving tumour-promoting inflammation. New vistas have emerged on molecules associated with M2 or M2-like polarization and its orchestration. Macrophage polarization has emerged as a key determinant of resolution of inflammation.

Reference(s)


[44] Proffered Paper: Osteopontin and Lactadherin Drive Pro-invasive Activation of Infiltrating Macrophages and Contribute to Glioma Progression

B. Kaminska1, A. Ellert-Miklaszewska1, P. Wniewrokski1, M. Kijewska1, M. Dabrowski1, K. Gabrusiewicz1, M. Lipko1, 1Nencki Institute of Experimental Biology, Cell Biology, Warsaw, Poland

Background: Microglia/macrophages infiltrating malignant gliomas support invasion, angiogenesis, extracellular matrix remodeling and immunosuppression that contributes to glioma progression. Molecules and signaling pathways that direct macrophages toward a pro-invasive phenotype are poorly known.

Material and Methods: Classical inflammation-related signaling pathways and global transcriptional responses were analyzed in primary and established glioblastoma cell lines, exposed to glioma conditioned medium (GCM) or lipopolysaccharide (LPS). To identify factors that re-program microglia protocytic analysis of the C6 glioma secretome was performed. For functional studies siRNAs/shRNAs or an integrin binding interfering peptide were used. Glioma cells stably expressing gene specific or control shRNA were generated and implanted to the striatum of Wistar rats. Tumor volume, macrophage infiltration/activation (staining for lba1 and arginase ) and angiogenesis (vWF staining) in tumor bearing brains were evaluated at day 17 after implantation. Quantification of gene expression in low and high grade human gliomas from the Canadian Brain Tumor Bank was performed by qPCR. Kaplan–Meier survival curves were acquired from the NCI Rembrandt depository.

Results: Tumor-driven programming of microglia is not associated with induction of inflammation-related signaling and gene expression but triggers specific genomic responses with induction of Id and c-Myc transcription factors and several markers of the alternative M2 phenotype. Proteomic analysis of glioma secretome identified osteopontin (Spp1) and lactadherin (Mig68) that activate integrin receptors and downstream focal adhesion kinase and PI-3K/Akt signaling in microglial cells. It increases cell motility, phagocytosis, supports proliferation and induces specific genes. Interference with integrin binding or osteopontin or lactadherin expression block microglial activation. Glioma cells depleted of osteopontin or lactadherin form significantly smaller tumors than controls, show reduced macrophage activation and angiogenesis in vivo. The expression of SPP1 is highly upregulated in glioblastoma multiforme samples. Kaplan–Meier survival curves showed inverse correlation between SPP1 expression and glioma patients survival.

Conclusions: Osteopontin and lactadherin (highly expressed in gliomas) via integrin receptors augment microglia motility, phagocytosis, proliferation and induce the alternative activation. Osteopontin supports microglia-dependent glioma invasion in vitro and in vivo. Supported by the grant N N301 786240.

[45] Control of Tumour Progression and Metastasis by Lymphocyte-Produced Cytokines

M. Karin1, 1University of California San Diego, Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, La Jolla CA, USA

Inflammation and immunity can interact with tumor development in more than one way. While chronic inflammation promotes tumor development, many tumors that do not arise in the context of underlying inflammation still exhibit an inflammatory microenvironment. Furthermore, in certain cases, inflammation may act to suppress anti-tumor immunity, but it can also be used to enhance the efficacy of cancer immunotherapies. Consequently, we need to learn much more about how inflammation and immunity affect tumor development. To study the pathogenic roles of tumor-elicted inflammation, we have used mouse models of prostate and breast cancers, two of the most common malignancies in advanced women, usually, which usually do not evolve in the context of underlying inflammation or infection. Yet, in both cases, we found that tumor-elicted inflammation plays a key role in promoting metastatic spread and in the case of prostate cancer, it contributes to the failure of androgen ablation therapy. Interestingly, in both types of cancer, metastasogenesis depends on the accumulation of activated iKB kinase a (IKKa) in the nuclei of primary cancer cells, where it acts both as an activator of chromatin modifiers that control all cycle progression and as a repressor of an anti-metastatic gene, called maspin. In both cases, IKKα, whose activation has also been observed in advanced human tumors, may be activated upon production within the tumor microenvironment of two members of the TNF family of cytokines:

lymphotokinin (LT)αβ and RANK ligand (RANKL). While the cells responsible for production of these cytokines during metastatic progression of prostate cancer remain to be identified, B cells were found to be a major source of LTαβ during development of castration resistant cancer. In breast cancer, however, the major culprits in metastatic progression are RANKL-producing regulatory T cells (Treg). Both in prostate and breast cancers, the recruitment of lymphocytes into the primary tumor is likely to depend on activation of myofibroblasts which produce a number of tumor promoting chemokines. LTαβ, RANKL, IKKα and the mechanisms responsible for myofibroblast activation, as well as chemokines they produce provide several new opportunities for therapeutic intervention.

Reference(s)

Sunday 8 July 2012 17:30–19:15 Symposium Cell Death/Autophagy

[46] Role of Autophagy in Cancer Metabolism
E.P. White1, 1The Cancer Institute of New Jersey, Rutgers University, New Brunswick, NJ

The cellular self-cannibalization process of autophagy is activated by stress and starvation to capture proteins and organelles and deliver them to the lysosomal compartment for degradation. The resulting breakdown products, such as amino acids and sugars, are then released into the cytoplasm where they can be recycled to sustain metabolism or used for generation of new biomass. Normal cells use autophagy to survive interruptions in growth factor and nutrient availability and to eliminate damaged proteins and organelles, the accumulation of which is toxic. Autophagy has a dual role in cancer. Loss of autophagy normal tissue results in damage, inflammation and genome instability resulting from failure of protein and organelle quality control that can promote cancer initiation. In this context autophagy is a tumor suppression mechanism, particularly in the liver. In tumors, autophagy is activated by stress in the microenvironment and supports cancer cell survival. Autophagy is also upregulated in established cancers and supports metabolism, stress survival, therapy resistance and tumorigenesis. In these contexts autophagy is tumor promoting. The role of autophagy in the settings of cancer initiation, progression and therapy, and its functional role in protein and organelle quality control and in metabolism will be discussed. Insights from autophagy modulation in genetically engineered mouse models for cancer, and in the context of cancer therapy, will be addressed.

[47] Regulation of Inflammation and Cell-Death Trough Interactions of RHIM-domain Protein Kinases With Caspase-8
D. Wallach1, T.B. Kang2, S.H. Yang3, B. Toth Cohen1, A. Rajput1, J.C. Kim1, A. Kovalenko1, 1Weizmann Institute of Science, Biological Chemistry, Rehovot, Israel, 2College of Biomedical and Health Science Konkuk University, Department of Biotechnology, Chungu, Korea

Caspase-8 was discovered as the proximal enzyme in the `extrinsic' cell-death pathway - one of the main mechanisms by which innate immune cells impose apoptotic cell death. This pathway is restricted by signaling for activation of NF-κB - transcription factors that also control inflammatory genes. Cell vulnerability to death induction by the extrinsic pathway is therefore likely to decrease in association with induction of inflammation. Conversely, once the extrinsic death pathway does become activated various proteins that signal for NF-κB activation are cleaved and thus inactivated, resulting in arrest of induction of inflammation. The apoptotic process itself restricts inflammation by safeguarding against release of damage-associated molecular patterns from the dying cell and by immunosuppressive effects of apoptotic cell membrane structures. Studies of recent years revealed that caspase-8 also blocks inflammation by additional mechanisms: it withdraws direct signaling for inflammation as well as signaling for necrotic death, and thus indirectly to inflammation, by death-receptors and by a number of pattern recognition receptors. These inhibitory effects request the association of caspase-8 with various different signaling complexes that affect a variety of different cellular functions. One common denominator to these different signaling complexes is that they all encompass the RHIM-domain containing protein kinase RIPK1. Some also contain the RHIM-domain kinase RIPK3. In all these complexes, the inhibitory effect of caspase-8 is associated with proteolytic cleavage of these kinases, which arrests signaling by these complexes and yields proteolytic fragments of the kinases that may act as anti-inflammatory! Consistent with these functions of caspase-8, in several transgenic mice models its deficiency was found to prompt severe inflammation. Recent advances in elucidating the mechanisms for the anti-inflammatory function of caspase-8 will be presented.

[48] Profivered Protein Bim Plays a Critical Role in Hodgkin Lymphoma Cells Induced by HDAC Inhibitor Givinostat and the Multikinase Inhibitor Sorafenib
S.L. Localiti1, A. Guidetti2, L. Cleris3, A.M. Gianni4, A. Anichini5, C. Carlo-Stella1, 1IRCCS Istituto Clinico Humanitas, Onco/Hematology, Rozzano (Milan), Italy, 2 Fondazione IRCCS Istituto Nazionale Tumori, Medical Oncology, Milan, Italy, 3 Fondazione IRCCS Istituto Nazionale Tumori, Experimental Oncology, Milan, Italy, 4 Fondazione IRCCS Istituto Nazionale Tumori, Human Tumors Immunobiology Unit, Milan, Italy

Introduction: A significant proportion of Hodgkin lymphoma (HL) patients refractory to first-line chemotherapy or relapsing after autologous transplantation are not cured with currently available treatments and require new treatments. Reversible acetylation mediated by histone deacetylase (HDAC) and the RAF/MEK/ERK pathway influence a broad repertoire of physiological processes, many of which are aberrantly controlled in HL cells. Since HDAC and RAF/MEK/ERK inhibitors are among the most promising targets these pathways using the HDAC inhibitor Givinostat (Iattarmaco S.p.A., Milan, Italy), and the RAF/MEK/ERK inhibitor Sorafenib (Nexavar, Bayer, Germany, EU) in order to investigate in vitro and in vivo the activity and mechanism(s) of action of this two-drug combination.

Material and Method: HL cell lines (HD-MYz, L-540 and HDLM-2) were used to investigate the effects of Givinostat and Sorafenib using in vitro assays analyzing cell growth and cell death. Western blotting (WB) analysis were performed to determine whether the two-drug combination affected MAPK, PI3K/AKT and HDAC pathways as well as apoptosis. The efficacy of Givinostat/Sorafenib combination was subsequently confirmed in a xenograft model in NOD/SCID mice.

Results and Discussion: While Givinostat and Sorafenib as single agents exerted a limited activity against HL cells, exposure of all HL cell lines to Givinostat/Sorafenib combination resulted in synergistic cell growth inhibition (70% to 90%). Upon Givinostat/Sorafenib exposure, HDLM-2 and L-540 cell lines showed significant levels of apoptosis (90% ± 2% and 96 ± 1%, respectively P < 0.0001) associated with severe mitochondrial dysfunction (up to 70%, P < 0.0001) and synergistic increase in generation of reactive oxygen species (ROS) (up to 60%, P < 0.0001). Apoptosis induced by Givinostat/Sorafenib combination resulted in modest processing of caspase-8, -9, -3, or cleavage of PARP and was not reversed by the pan-caspase inhibitor Z-VADfmk, suggesting a major contribution of caspase-independent rather than caspase-dependent apoptosis. In all cell lines, WB analysis showed that cytotoxic and anti proliferative effects in Givinostat/Sorafenib-treated HL cells were associated with severe dephosphorylation of MAPK and PI3K/AKT pathways and significant increase in H3 and H4 acetylation due to HDAC inhibition. Interestingly, these events were associated with a pronounced time-dependent up-regulation of the BH-3 only protein Bim, and down-regulation of Mcl-1. In vivo Givinostat/Sorafenib treatment significantly reduced the growth of HD-MYz nodules, resulting in an average 65% tumor growth inhibition (P < 0.001), compared to single treatments, in the absence of any toxicity.

Conclusion: Both in vitro and in vivo, Givinostat/Sorafenib combination resulted in potent anti-HL activity. These results warrant clinical evaluation in HL patients.

[49] Regulation of Apoptosis by the Bcl-2 Family
S. Cove1, K. Campbell1, C. Vandenbergh2, K. Mason2, R. Bizard3, C. Scott4, A. Ferguson1, D. Huang1, J. Adams1, S. Strickland, 1The Walter and Eliza Hall Institute for Medical Research, Molecular Genetics of Cancer Division, Melbourne, Australia, 2The Walter and Eliza Hall Institute for Medical Research, Cancer and Haematology Division, Melbourne, Australia

Background: Impaired apoptosis is a critical step in the development of cancer and a major impendence to effective therapy. Bcl-2, the oncprotein activated by chromosome translocation in human follicular lymphoma, inhibits cells from undergoing apoptosis in response to many cytotoxic agents. Several close relatives (Bcl-xL, Bcl-w, Mcl-1 and A1) are also anti-apoptotic but others (Bax and Bak) are instead pro-apoptotic, despite strong structural and functional homology. More distant pro-apoptotic family members, the ‘BH3-only proteins’ are largely unrelated, apart from the signature BH3 (Bcl-2 homology region 3) domain that is essential for their killing function. There is currently great interest in developing small molecules that mimic the action of Bcl-3-only proteins as novel cancer therapeutics.

Materials and Methods: Using transgenic mice, we have compared the impact of over-expression of Bcl-2 and its most divergent anti-apoptotic relative, Mcl-1,
on normal haemopoiesis, tumour development and tumour treatment. We have also investigated the impact of loss of specific BH3-only proteins on tumour development in mouse models of lymphoid and myeloid malignancy and in responsiveness to therapy. We are testing the efficacy of the BH3 mimetic ABT-737, which binds to Bcl-2, Bcl-xL and Bcl-w, for treating different haemopoietic malignancies in vitro and in immune-competent mice.

Results and Conclusions:
– Bcl-2 and Mcl-1 are potent oncoproteins for lymphoid cells.
– Over-expression of Mcl-1 promotes malignant transformation of haemopoietic stem and progenitor cells.
– Certain BH3-only proteins serve as suppressors of Myc-driven lymphomagenesis.
– Different BH3-only proteins are triggered by different cytotoxic agents.
– Recent studies, including our own, suggest that BH3 mimetics like ABT-737 will be highly effective agents for treating haemopoietic malignancies having high levels of Bcl-2 and low levels of Mcl-1.
The Rise of Bioinformatics in Data-driven Cancer Research

P. Bork1. 1European Molecular Biology Laboratory, Heidelberg, Germany

The development of computational tools and databases started already in the 70s, mostly for sequence and structural analysis. The advent of the personal computer enabled propagation in the 80s with a further boost provided by the internet in the early 90s. With an increasing ‘omics’ mindset during the 1990s, the field increased and diversified considerably, a tendency that still holds today, amplified by the exponentially increasing amounts of data that are being generated in biology and medicine. With respect to cancer, early bioinformatics contributions were the prediction of function for cancer genes, analysis of GWAS and transcription data as well as network analysis. Currently NGS data revolutionize cancer research and are essentially relying on data handling and digestion with an increasing network modeling component. Diverse data types are currently been explored for biomarker detection from blood to stool, with the hope for early diagnosis or prognosis and eventually better treatment. Here I introduce into some of the concepts, tools and resources in Bioinformatics and will illustrate this with current research utilizing the human microbiome for biomarker discovery.

Statistical Challenges in the Development of Reliable and Clinically Meaningful Biomarkers

L. McShane1. 1U.S. National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis

A better understanding of tumor biology through identification of specific biological characteristics of tumor and host that predict tumor behavior and responsiveness to therapy will be essential for the discovery of new therapies and for optimization of cancer care for individual patients. Although thousands of correlative studies relating individual biomarkers or high-dimensional biomarker profiles to clinical characteristics or clinical outcome have been published, only rarely have these findings led to clinically useful biomarker-based tools. Many correlative studies are conducted without clearly specified hypotheses, have poor statistical designs, use assays that are not standardized or lack reproducibility, and often employ inappropriate or misleading statistical analysis approaches. Common statistical problems include underpowered studies or overly optimistic reporting of effect sizes and significance levels due to multiple testing, subset analyses, cutpoint optimization, and model overfitting. For studies aiming to assess a biomarker’s ability to guide treatment decisions, the importance of appropriate control groups and the potential for confounding of prognostic and predictive effects have been underestimated. Compounding these problems, many correlative studies have not been reported in a rigorous fashion, and published articles often lack sufficient information to allow adequate assessment of the quality of the study or the generalizability of the results. Suggestions are provided for how to avoid some of the common statistical pitfalls, how to identify problematic studies, and how to report correlative studies to maximize the interpretability and usefulness of the results.

The use of Metabolomics to Discover Novel Metabolic Networks and new Targets for Cancer Treatment

E. Gottlieb1. 1Cancer Research UK Beatson Institute for Cancer Research, Apoptosis and Tumour Metabolism Laboratory, Glasgow, United Kingdom

The extent to which metabolism plays a role in tumorigenesis cannot be overstated and drugs that selectively target these processes are likely to at least delay, if not halt tumour progression. Our work utilizes analytical chemistry and system biology approaches to study metabolic transformation.

Monday 9 July 2012 08:00–08:50

Educational Lectures

Monday 9 July 2012 09:00–09:45

Meet the Expert
tissue. More translational studies are needed to validate innovative imaging signals in patients with various tumor entities. It is expected, that molecular imaging will play an important role to enable individualized therapeutic strategies based on the visualization of tumor biology in human.

Background and Methods: Based on systematic reviews conducted at independent academic institutions in the USA, UK and continental Europe, an independent expert panel convened by WCRF International estimated that between about a third and a quarter of the commonest cancers worldwide could be prevented through improved patterns of diet and activity, and healthy body weight.

Results: The recommended dietary pattern, high in fibre-rich plant foods and low-energy, relatively unprocessed foods, and low in red and processed meat, alcohol, salt and high energy foods, together with increased levels of activity, will also prevent other common noncommunicable diseases, including obesity, diabetes and cardiovascular disease. This evidence has been combined into a single database, and is being updated continuously into the future. Based on separate reviews of the determinants of people’s behaviour, the panel concluded that people’s behaviour is shaped by their physical environment, and their social and economic circumstances as well as their personal knowledge, beliefs and attitudes. They concluded that education-based health promotion alone was unlikely to achieve the recommended changes, and broader policy action was needed to make the environments that determine people’s choices more conducive to health. They emphasized the evidence according to their confidence in it, and its likely impact, and made evidence-based recommendations to nine groups of actors across the whole of society. These actors include national and international governmental and non-governmental organisations, industry, health and other professionals, schools, workplaces and other institutions, the media, as well as people themselves.

Conclusions: Coherent action by all actors is needed to be effective, and leadership should come from health professionals and government. These two reports, published in 2007 and 2009 (Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective; and Policy and Action for Cancer Prevention), are the most comprehensive and authoritative reviews of this area.

Monday 9 July 2012 10:15–12:00

Symposium
Mouse Models

Analyzing Inflammation-induced Liver and Skin Cancers in AP-1(Fos/Jun)-dependent Mouse Models

E.F. Wagner, BBA Foundation-CNIO Cancer Cell Biology Programme, Spanish National Cancer Research Centre (CNIO); Madrid, Spain

Inflammation plays a role in the development of several chronic diseases, such as liver cirrhosis and cancer. Understanding the molecular pathways involved in the development of these diseases is of great importance. The results of the present study will be presented.

Reference(s)

Proffered Paper: Next Generation RNAi Mouse Models for Drug Studying Therapy Response and Resistance in Mouse Models

M. Wiseman, 1 WCRF International, London, United Kingdom

Background: It has been shown that in vivo methods are powerful tools in determining the functional role of any gene. The development of transgenic mice has allowed the induction of single mutants, but was limited by the necessity to occasionally perform a targeted disruption of the gene in embryonic stem cells. The recent advent of RNAi has enabled the rapid development of a system that can be used to produce multi-allelic mouse models. Following the initial successes of single-gene gene targeting, the next generation of mouse models will have to be multi-allelic knockouts. In the present study, we have shown that this can be achieved using the Platform for Generation of Mutants (PGM) to generate multi-allelic mouse models.

Results: The Platform for Generation of Mutants (PGM) has been recently used to create multi-allelic mouse models. This system enables the rapid generation of a large number of knockout mice with the same genetic background and allows the study of gene function in a variety of different tissues and organs. The PGM has been used to create multi-allelic mouse models for a variety of different genes and has been shown to be a powerful tool for the study of gene function in vivo.

Conclusions: The PGM is a powerful tool for the generation of multi-allelic mouse models and has been shown to be a valuable resource for the study of gene function in vivo. The PGM has been used to create a variety of multi-allelic mouse models and has been shown to be a powerful tool for the study of gene function in vivo. The PGM is a valuable resource for the study of gene function in vivo.

Materials and Methods: The PGM is a powerful tool for the generation of multi-allelic mouse models and has been shown to be a valuable resource for the study of gene function in vivo. The PGM has been used to create a variety of multi-allelic mouse models and has been shown to be a powerful tool for the study of gene function in vivo. The PGM is a valuable resource for the study of gene function in vivo.

Validation of Therapeutic Targets in K-Ras Driven Lung and Pancreatic Tumors

M. Barbaric, 1 Centro Naz. de Inv. Oncologicas, Cnio, Madrid, Spain

Background: K-Ras oncoproteins have been implicated in about one fifth of all human cancers including those with the worse prognosis, such as non-small cell lung carcinoma (NSCLC), pancreatic ductal adenocarcinoma (PDAC) and colorectal carcinoma (CRC).

Materials and Methods: We have used genetic engineering strategies to develop genetically modified mouse (GEM) models for each of these tumor types that closely recapitulate the natural history of these human neoplasias. K-Ras driven NSCLC and PDAC models were performed using different strains of mice carrying germ line or conditional knock out mutations in loci encoding potential therapeutic targets. Targets were ablated by genetic means either at the time of tumor initiation or, whenever possible, once the tumor has been generated. Then, we followed the fate of the tumor with the use of transgenic models. This genetic-based strategy has significant advantages over classical pharmacological studies since it does not rely on the quality of the drug/inhibitor and the observed effects are mechanism-based and not off-target effects.
Results: In the NSCLC model, we have targeted the individual members of the Raf/Mek/Erk kinase cascade, the primary signaling pathway involved in mediating K-Ras signaling. Ablation of Mek1 or Mek2 in K-Ras oncogene expressing lung cells had no significant effect due to compensatory activities. However, elimination of both Mek kinases completely blocked tumor development. Unfortunately, the systemic elimination of Mek1/2 kinases was found to be incompatible with adult life. In the case of the Raf kinases we did not observed a similar compensatory activity. Whereas ablation of the B-Raf kinase had no significant effect on tumor development, c-Raf expression was absolutely essential for the onset of NSCLC. Interestingly, concomitant elimination of c-Raf and B-Raf in adult mice had no deleterious consequences for normal homeostasis.

In the PDAC model, we have shown that tumor development in adult mice requires an inflammatory insult (e.g. pancreatitis), which contributes to tumor progression by abrogating the senescence barrier characteristic of benign PanIN lesions. Attenuation of the inflammatory response accelerates tissue repair and thwarts PanIN expansion. We have also used this model to ablate potential therapeutic targets involved in K-Ras signaling. Early clinical studies have suggested a therapeutic benefit of Erlotinib, an EGF receptor inhibitor, in pancreatic adenocarcinoma patients. These results were unexpected since clinical evidence in lung and colon tumors has illustrated that multifaceted activation of EGF receptors is mutually exclusive with the presence of K-RAS oncogenes. However, our results indicate that the effect of Erlotinib in PDAC tumors may have a mechanistic base. Indeed, activation of EGF receptors expression is one of the earliest events in the injured pancreas. More importantly, genetic evidence revealed that EGF receptors are essential for tumor development even in the context of pancreatitis and loss of p16INK4a/p19ARF tumor suppressors Only loss of TPS3 relieved the need to maintain EGF receptor signaling during PDAC development. Yet, ablation of these receptors considerably increased survival. Explants derived from these tumors were inhibited by a combination of PI3Kinase inhibitors and Stat3 knock down.

Conclusions: Our results in the NSCLC GEM model strongly suggest that c-Raf is a target with potential therapeutic value. Likewise, our observations in the PDAC GEM model indicate that successful treatment of PDAC tumors may require inhibition of at least four distinct signaling cascades including those driven by K-RAS oncogenes, EGF receptors, PI3Kinase and Stat3. The information derived from these studies should provide the experimental bases to initiate novel drug discovery programs that may result in more effective therapeutic approaches to treat K-RAS-driven NSCLC and PDAC in human patients.

Monday 9 July 2012
10:15–12:00
Symposium
Stem Cells

[60] Cancer-initiating Cells in Glioblastoma
No abstract received.

[61] Interactions Between Breast Cancer Stem Cells and their Niche: Govern Metastatic Colonization of the Lung
I. Malanchi1, A. Santamaria-Martínez1, E. Susaondo1, H. Peng1,2, H.-A. Lehn1, J.-F. Delatour1, J. Huelken1, École Polytechnique Fédérale de Lausanne (EPFL), ISREC (Swiss Institute for Experimental Cancer Research) and National Center of Competence in Research (NCCR) ‘Molecular Oncology’, Lausanne, Switzerland, 2 Nanfang Hospital, Department of Otorhinolaryngology, Head and Neck Surgery, 1 University Institute of Pathology, CHUV, University of Lausanne, Switzerland, 3 Department of Gynecology and Obstetrics, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

Metastatic growth in distant organs is the major cause of cancer mortality. We now identify the niche as a key element for the initiation of breast cancer metastasis: first, a small population of breast cancer stem cells that are critical for metastatic colonization, i.e. the initial expansion of cancer cells at the secondary site. Second, we find stromal niche signals to be crucial for facilitating cancer cells invasive potential to the extracellular matrix (ECM) component peroxin (POSTN) to be expressed by fibroblasts in the normal tissue and in the stroma of the primary tumour. Importantly, infiltrating tumour cells need to induce stromal POSTN expression in the secondary target organ lung to initiate colonization. This ECM protein is required to allow cancer stem cell maintenance and blocking POSTN function prevents metastasis. POSTN recruits Wnt ligands and thereby increases Wnt signalling in cancer stem cells. We suggest that the education of stromal cells by infiltrating tumour cells is a step in metastatic colonization and that preventing de-novo niche formation may represent a novel treatment strategy against metastatic disease.

Monday 9 July 2012
10:15–12:00
Symposium
Molecular Insights into Invasion and Drug Resistance

[62] Proffered Paper: Regression of Metastatic Melanoma by Targeting Melanoma Stem Cells
H. Abken1, P. Schmidt2, M. Schlaak3, A.A. Hornbach3, C. Bangard3, P. Kurschat2, P. Zignor2. 1Klinik für Innere Medizin, Zentrum für Molekulare Medizin Köln, Köln, Germany, 2 Klinik für Innere Medizin, Köln, Germany, 3 Klinik für Dermatologie, Köln, Germany, 4 Institut für Radiologie, Köln, Germany

Background: Current paradigms in cancer therapy attempt to eliminate all malignant cells of a tumor lesion, the cancer stem cell (CSC) paradigm, however, predicts that tumors are maintained by a few, so far less identified cancer stem cells.

Material and Method: Human melanoma biopsies were transplanted into immune deficient mice and subsequently treated by adoptive transfer of T cells engineered with a chimeric antigen receptor targeting melanoma cell subsets.

Results: Here we demonstrate that specific elimination of a less than 2% subset of melanoma cell eradicates transplanted human melanoma biopsies in a mouse model without targeting the bulk of tumor cells. The melanoma cell subset is selectively and specifically eliminated from established tumor lesions by adoptive transfer of cytotoxic T cells redirected by a chimeric antigen receptor. Targeted elimination of the minority of CD20+ melanoma cells eradicated melanoma lesions in the long-term despite the non-targeted tumor cell mass. Targeting of any random cancer cell subset was not effective.

Based on these pre-clinical data we attempted to eliminate those cells in a patient with progressing, chemotherapy-refractory metastatic melanoma by lesional injections of the anti-CD20 therapeutic antibody rituximab. Although the frequencies of CD20+ melanoma cells within the tumor lesions were initially about 2% and the bulk of tumor cells did not express CD20, rituximab treatment produced lasting remission accomplished by a decline of the melanoma serum marker S-100 to physiological levels. Apart from B cell elimination and decline in gammaglobulin levels, no grade 3/4 toxicity related to treatment was observed.

Conclusion: Data provide the first clinical evidence of CD20+ melanoma sustaining cells and highlight the potency of selective cancer cell targeting in the treatment of melanoma.

[63] Circulating Metastasis-initiating Cells in Breast Cancer
I. Baccielli1,2, S. Riehdorf1, M. Wallwieder1, C. Klein1,2, K. Pantel1, W. Weichert1, A. Schneeweiss1, A. Trumpp1,2, 1DKFZ – German Cancer Research Center and Hi-STEM, Stem Cells and Cancer, Heidelberg, Germany, 2 Hi-STEM, Heidelberg, Germany, 3 Universitätsklinikum Hamburg-Eppendorf, Tumor Biology, Hamburg, Germany, 4 Nationales Centrum für Tumorerkranungen, Heidelberg, Germany, 5 Pathologisches Institut d. Universität Heidelberg, Heidelberg, Germany

Metastasis is the foremost cause of cancer-related deaths. Metastatic spread is a complex process initiated by the dissemination, seeding and engraftment of malignant cells in sites distant to the primary tumor. It has been hypothesized that metastasis-initiating cells (MICs) are present within circulating-tumor-cells (CTCs) in the blood stream of carcinoma patients. Indeed, the presence of CTCs in metastatic patients correlates with decreased overall-survival in several malignancies, including breast cancer. Although these clinical data are consistent with the hypothesis that CTCs contain MICs, their existence, phenotype and activity has never been demonstrated. We present data showing that as low as 1900 CTCs were able to induce metastases in mice. Transplantation of primary patient blood derived CTCs induced metastatic growth in bones and liver, demonstrating the presence of MICs. FACS analysis of primary patient EPCAM+CTCs revealed heterogeneous inter-patient expression of the metastasis-promoting signaling receptors CD44, CD47 and MET. While the percentage of EPCAM+CD44+CD47+MET+ CTCs in patient blood varied between 1.4 and 44%, metastases both from the original patient and those derived experimentally from CTCs showed high levels of all three receptors. The data provide a first demonstration that EPCAM+CTCs express CD44, CD47 and MET and contain MICs, providing a molecular basis for the design of diagnostic tools to detect MICs and for developing rational-based approaches to target metastasis in breast cancer.
Pancreatic Cancer Therapies

D. Tuveson, D. Haber, S16 of clinical trials to probe the relevance of such findings in patients. Hypoxic necrosis and response. Several of our observations serve as the basis for the tumour microenvironment to increase the efficacy of gemcitabine. Additionally, gamma-secretase inhibition cooperates with gemcitabine to cause chemotherapy. We have now extended our observation to other agents that target the tumour microenvironment to increase the efficacy of gemcitabine. One of these, nab-Paclitaxel, increases gemcitabine levels not by enhancing drug delivery, but rather by targeting cyclin-dependent kinase for destruction. Additionally, gamma-secretase inhibition cooperates with gemcitabine to cause hypoxic necrosis and response. Several of our observations serve as the basis of clinical trials to probe the relevance of such findings in patients.

Proffered Paper: PTEN Phosphorylation by Fibroblast Growth Factor Receptors and SRC Mediates Response to Epidermal Growth Factor Receptor Inhibitors in Glioblastoma

F. Furnari, T. Trentini, H. Zhou, S. Marie, P. Mischel, W. Cavenee. Ludwig Institute-UCSD, La Jolla, USA, 2 Ludwig Institute-University of Sao Paulo, Sao Paulo, Brazil, 3 University of California Los Angeles, Los Angeles, USA, 4 Ludwig Institute-UCSD, Medicine, La Jolla, USA

Background: Glioblastoma multiforme (GBM) is the most aggressive of the astrocytic malignancies and the most common intracranial tumor in adults. Although the epidermal growth factor receptor (EGFR) is overexpressed and/or mutated in at least 50% of GBM cases and is required for tumor maintenance in animal models, EGFR inhibitors have thus far failed to deliver significant responses in GBM patients.

Material and Methods: Mass spectrometry was used to identify PTEN tyrosine phosphorylation sites and a phospho-specific antibody against one such site (Y240), was developed. This antibody was used to study PTEN tyrosine phosphorylation in cell lines and primary tumor samples from GBM patients. Wild type PTEN and a non-phosphorylated tyrosine 240 to phenylalanine (Y240F) mutant were used to define the role of Y240 phosphorylation in promoting EGFR tyrosine kinase inhibitor (TKI) resistance.

Results: One inherent TKI resistance mechanism in GBM is the co-activation of multiple receptor tyrosine kinases (RTKs) which generates redundancy in activation of the phosphatidylinositol-3′-kinase (PI3K) signaling. Here we identified a novel mechanism by which fibroblast growth factor receptors (FGFRs) and src family kinases (SKFs) impact on PI3K signaling in GBM by phosphorylating PTEN at a conserved tyrosine residue, Y240. Phosphorylation of Y240 was associated with shortened overall survival and resistance to EGFR TKIs in GBM patients, and played an active role in mediating resistance to EGFR inhibition in vitro. Experimentally, a non-phosphorylated Y240F mutant allele of PTEN potently sensitized cells expressing a constitutively active mutant of EGFR (EGRFVal320Met) to erlotinib. In contrast, activation of FGFR signaling promoted resistance to erlotinib in GBM cells expressing wild type PTEN, concordant with phosphorylation of PTEN and activation of downstream signaling.

Conclusions: Our findings identify a novel signaling connection between FGFRs and PTEN and provide a mechanistic link between PTEN regulation and drug resistance, suggesting that blocking PTEN phosphorylation by suppression of SRC and/or FGFR activity represents a potential strategy to re-sensitize tumors to EGFR inhibitors.

Molecular Characterization of Circulating Tumour Cells

D. Haber, 1 Massachusetts General Hospital Cancer Center, MGH Cancer Center, Boston MA, USA

Circulating tumor cells (CTCs) are shed into the bloodstream from primary epithelial cancers and their metastatic deposits. Although extraordinarily rare among normal blood components, they provide a unique opportunity to non-invasively sample tumor cells, monitor their response to therapy and potentially predict the emergence of drug resistance. Nowhere is this more critical than in the new field of targeted cancer therapies, where continuous analysis of tumor genotypes may allow rational adjustments of treatment regimens, ultimately achieving long term control of cancer. While promising, the analysis of CTCs is complicated by significant technological hurdles, including very rare cell detection within a complex fluid, high throughput imaging of cells expressing variable markers, and molecular analysis of incompletely purified heterogeneous cell populations. To address these challenges, our team of engineers, molecular biologists and clinicians has devised a series of microfluidic devices capable of capturing CTCs with high efficiency, optimized parameters for semi-automated imaging and quantitation, and initiated molecular genetic and digital gene expression analyses of captured cells. Studying lung, prostate and breast cancers, we find that CTCs can be detected in some localized invasive cancers as well as in metastatic cases. The molecular markers identified in CTCs are highly correlated with those of simultaneously sampled tumors, but may occasionally differ from those of primary tumors resected years before the development of metastatic disease. Certain features, such as proliferative index of CTCs (measured by Ki67 staining) differ dramatically between different clinical stages of disease. Mutations conferring acquired resistance to targeted agents can be detected within CTCs and the emergence of drug resistance in CTCs increases in concert with the advent of clinical drug resistance. Finally, RNA sequencing approaches may be used to generate digital expression profiles of CTC-enriched populations, providing confirmation as to their cell of origin, as well as identifying potential transcript that are upregulated in CTCs and may contribute to their metastatic propensity. Together, the molecular analysis of CTCs offers the potential to monitor drug responses in clinical trials, and also to identify potential new therapeutic targets aimed at suppressing cancer metastasis.

Targeted Cancer Therapy Development

A. Ulrich, Max-Planck Institute for Biochemistry, Department of Molecular Biology, Martinsried, Germany

For the past years we have investigated various aspects of signaling systems in tumor cells in order to identify critical switch points in the pathophysiologial process that results in malignancy. These efforts aim at the selective blockade of abnormal, disease-promoting signaling mechanisms by monoclonal antibodies, or small molecule kinase inhibitors. This strategic approach began with the cloning of the EGF receptor cDNA and the related receptor HER-2/neu and translated the animal oncogene concept into target-directed personalized therapy of human cancer. This work yielded the first specific oncogene target-based, FDA-approved (1998) personalized, therapeutic agent, “Herceptin”, for the treatment of metastatic breast cancer of a subgroup of individually defined patients. Earlier and subsequent “target-driven drug development” efforts that employed various genomic analysis strategies led to the cancer therapies that are based on EGFR, HER3, FGFR4, Axl/Un and Flik-1/VEGFR2 as critical signaling elements in tumor progression. The latter served, in cooperation with SUGEN Inc./Pharmacia/Pfizer, as basis for the development of SU1248/Sunitinib. The drug discovery process that started with Herceptin and led to Sunitinib represents a prototypical example for the adaptation of cancer therapeutics from highly specific to multi-targeted drugs.

While all novel cancer therapies target genetic alterations in tumor tissues innovative strategies are aimed at investigating the contribution of germ line determinants of the patient to disease progression and therapy response. One example is the common polymorphism at codon position 388 in the human FGFR4 gene of which the Arg388 allele represents a target for the development of an FGFR4 dependent cancer therapy development. Current findings and their consequences for patient-specific cancer therapy will be presented.
We have demonstrated that mRNAs can exert a biological activity that is independent of the protein for which they encode. Since this phenomenon is dependent on sequences contained in RNA transcripts, this phenomenon applies to protein-coding genes, pseudogenes, of which 19,000 have been predicted in the genome as well as long non-coding RNAs that have been recently identified to be pervasive in the cell and in biology. This new function is brought about by the ability of mRNAs – and RNAs in general – to bind and sequester microRNA molecules. microRNAs specifically repress the expression levels of many genes and have consequently been shown to play important roles in diseases including cancer. We show that any messenger RNA and RNA molecule can sequester microRNA molecules acting as a “competitive endogenous RNAs”. We therefore term them ceRNAs. Given that microRNAs can bind to multiple mRNAs, we argued that mRNAs compete for the binding to a given microRNA. We originally tested this hypothesis and proved its validity by studying the interaction between the mRNA encoding for the PTEN tumor suppressor gene and its closely related pseudogene, PTPN1, which we show to act as a tumor suppressor through this new mechanism. We therefore identify PTENP1 as well as the several thousands uncharacterized RNA molecules as potential human disease genes.

We have now expanded this analysis to the whole transcriptome. Our findings therefore define a new biological dimension that will allow for the rapid identification and functional characterization of new disease genes.

Monday 9 July 2012 15:15–16:00
Carcinogenesis Young Investigator’s Award Lecture

[70] Immune Surveillance of Senescent Cells: Biological Significance in Cancer- and Non-cancer Pathologies

L. Zender1,2, - Helmholtz Centre for Infection Research, Braunschweig, Germany, 1University of Mainz, Department of Neurosurgery, Mainz, Germany

Upon the aberrant activation of oncogenes, normal cells can enter the cellular senescence program, a state of stable cell-cycle arrest, which represents an important barrier against tumor development in vivo. Senescent cells communicate with their environment by secreting various cytokines and growth factors, and it has become clear that this ‘secretory phenotype’ can have pro- and anti-tumorigenic effects. Recent work from our laboratory showed that premalignant, senescent murine hepatocytes are recognized and cleared through an antigen-specific immune response and that this immune response, designated as ‘senescence surveillance’ is crucial for tumor suppression in the liver (Kang et. al. Nature 2011). It is an emerging concept that immune responses against senescent cells have a broader biological significance in different cancer – as well as non-cancer pathologies and current data suggest that distinct immune responses are engaged to clear senescent cells in different disease settings. In my talk I will discuss different examples how immune responses against senescent cells are involved to restrict disease progression in cancer- and non-cancer pathologies.

Monday 9 July 2012 17:30–19:15
Symposium
Targeted Therapies / Signalling Pathways

[71] Specific Fluorescent Probes for Gioma Cells in Living Cells and Tissue – Tools for Guided Resection

D.J. Arndt-Jovin1, S. Kantelhardt2, M. Bruchez2, A. Giese4, T.M. Jovin5, - Max Planck Institute for Biophysical Chemistry, Laboratory for Cellular Dynamics, Göttingen, Germany, 1University of Mainz, Neurosurgery, Mainz, Germany, 2Carnegie Mellon University, Department of Chemistry, Pittsburgh PA, USA, 4University of Mainz, Department of Neurosurgery, Mainz, Germany

Background: The current therapy of malignant gliomas is based on surgical resection, radio-chemotherapy and chemotherapy. Retrospective case-series have highlighted the significance of the extent of resection as a prognostic factor predicting the course of the disease. Complete resection in low-grade gliomas showed high probe specific, even with low-grade tumor for which no enhanced MRI image could be obtained. Tumor cells were visualized from the microscopic to single cell level with contrast ratios (tumor to brain) as high as 1000:1. QDs pose a problem for use in patients with low-grade gliomas since they contain both Cd and Se. More recently we have been developing other highly fluorescent probes utilizing dyes (branched fluoroargent dyes). Results will be presented for cell culture systems using both aptamers and affibodies for specific targeting of the glioma cells. Comparisons are made with the previously developed QD-probes.

Conclusions/Significance: The ability of the targeted probes to clearly distinguish tumor cells expressing growth factor receptors in low-grade tumor biopsies demonstrate the great potential of fluorescent probes for in situ localization of tumor boundaries. The new probes have potential for direct clinical application and are presently being tested with tumor material. We propose that the future application of specifically targeted fluorescent particles during surgery could allow intraoperative guidance for the removal of residual tumor cells from the resection cavity, thereby increasing patient survival.

Targeting JAK-STAT Signaling for Cancer Therapy

R. Jove1, - City of Hope Comprehensive Cancer Center, Molecular Medicine, Duarte, USA

Persistent signaling through the Janus Kinase (JAK) and Signal Transducers and Activators of Transcription (STAT) protein families is frequently associated with malignant progression of tumor cells. The JAKs, including JAK1, JAK2, are activated by autocrine or paracrine production of cytokines (eg, IL-6) and growth factors (eg, PDGF) in the tumor microenvironment. The activated JAKs in turn phosphorylate specific tyrosine residues in the STATS, particularly STAT3 and STAT5 in tumors, thereby inducing nuclear translocation of the STATs. In the nucleus, STATs regulate the expression of genes that contribute to malignant progression, including inhibition of tumor cell apoptosis. Numerous studies have established that inhibition of JAK-STAT signaling results in suppression of tumor growth, thus implicating JAK-STAT signaling as a promising target for cancer therapy. In this study, small-molecule inhibitors of JAK kinase activity were used to block phosphorylation of STAT proteins. These JAK inhibitors were used in model human tumor cell lines in culture as well as in human tumor xenograft models in mice. The effects of the JAK kinase inhibitors on tumor growth were monitored in several human tumor models, including both solid and liquid tumors. Our results demonstrate that JAK kinase inhibitors effectively block JAK-STAT3 signaling in cultured tumor cell lines. This inhibition was demonstrated by blockade of STAT3 phosphorylation, nuclear translocation, and DNA-binding activity. Importantly, JAK kinase inhibitors also blocked STAT3 signaling in human xenograft tumors, associated with suppression of tumor growth in mice. The critical role of STAT3 in this response to the JAK inhibitors was validated using genetic approaches including shRNA against STAT3 and mutational activation STAT3. Thus, inhibitors of JAK family kinases show promise as effective blockers of downstream STAT3 signaling pathways in tumors. This inhibition of JAK-STAT3 signaling in the tumor microenvironment is associated with suppression of human tumors in xenograft mouse models. In sum, JAK family kinase inhibitors show promising antitumor activity in human tumors harboring activated STAT3, including solid tumors and blood malignancies.

[72] Proffered Paper: Gial Cell Line-derived Neurotrophic Factor (GDNF) Signaling as a Target for Endocrine Therapy Resistance in Breast Cancer

A. Morandi1, Q. Gao1, P. Francia1, A. Mackay2, J.S. Reis-Filho3, M. Zvelebil1, M. Dowsett3, L.A. Martin3, 1Plaza-Menacho, 2C.M. Isacke3, - The Institute of Cancer Research, Breakthrough Breast Cancer Research Centre, London, United Kingdom, 1London Research Institute Cancer Research UK, Structural Biology Laboratory, London, United Kingdom

Introduction: Understanding the complex molecular mechanisms that underlie resistance to endocrine therapy is a major challenge in breast cancer research. We previously showed that GDNF/FRET signaling plays an important role in estrogen receptor (ER) positive breast tumors and as a prognostic factor predicting the course of the disease. Complete resection in low-grade gliomas that show no MRI-enhanced images are especially difficult. Our aim is to develop robust, specific, new fluorescent probes for glioma cells that are easy to apply to live tumor biopsies and could identify tumor cells from normal brain cells at all levels of magnification. A procedure will be developed for clinical use.

Methodology and Principal Findings: In earlier studies we employed brightly fluorescent, photostable quantum dots (QDs) to specifically target the epidermal growth factor receptor (EGFR) that is upregulated in many gliomas. Living glioma and normal cells or tissue biopsies were incubated with QDs coupled to EGF and/or monoclonal antibodies against EGFFR for 30 minutes, washed and imaged. The results obtained from cell-culture, animal model and ex vivo human glioma samples of both types showed that the QDs pose a problem for use in patients with low-grade gliomas since they contain both Cd and Se. More recently we have been developing other highly fluorescent probes utilizing dyes (branched fluoroargent dyes). Results will be presented for cell culture systems using both aptamers and affibodies for specific targeting of the glioma cells. Comparisons are made with the previously developed QD-probes.
IC182,780 (to discriminate between ER dependent and ER independent GDNF-regulated genes) and were subject to gene expression profiling. We defined a GDNF-responsive genes set (GROG) of 83 up- or down-regulated genes. After filtering out 16 proliferation related genes, the GROG was correlated with clinical and pathologic variables and with patient outcome. In vitro models of AI sensitivity and resistance were then used to elucidate the role of GDNF signaling in response to AIs.

Results and Discussion: In 3 independent datasets of profiled human breast tumors, the GRGS profile was correlated with luminal B breast cancers, a number of other prognostic factors and a shorter time to a poor outcome event. Importantly, the GRGS was an independent indicator of disease outcome. Most of the genes in the GROG were also associated with poor patient outcomes in independent datasets. Our study therefore demonstrated that GRGS could be used to select a subset of patients who would benefit from adjuvant endocrine therapy.

Conclusion: GDNF/RET activation is associated with enhanced breast cancer aggressiveness and tumor cell growth in the absence of estrogen. The data presented here support the GDNF/RET signaling pathway as a potential therapeutic target, particularly in breast cancers resistant to endocrine therapies.

IPM and CMI joint senior authorship.

Targeting the Immune System to Cancer

A. Levitzki1, A. Shir1, O. Berhani1, Y. Langut1, M. Zigler1, E. Ben-Ezri1, S.18

We have investigated the variability of primary breast cancer in its response to endocrine therapy. This is necessary. Recent evidence indicates that tumour endothelium may play a pivotal role in the tumour microenvironment. We have performed numerous studies investigating the role of tumour endothelium in the response of patients to endocrine therapy. The first study was to investigate the role of tumour endothelium in the response of patients to endocrine therapy. The second study was to investigate the role of tumour endothelium in the response of patients to endocrine therapy. The third study was to investigate the role of tumour endothelium in the response of patients to endocrine therapy. The fourth study was to investigate the role of tumour endothelium in the response of patients to endocrine therapy.

Cancer researchers agree that one has to find ways to harness the immune system to kill cancer cells with no collateral damage and reinstate immune surveillance. We suggest using Polyinosinic/Cytidylic chemical vectors guided by a ligand to the surface of cancer cells that over-express a protein, which internalizes upon the binding of the ligand. As a consequence, large amount of the synthetic dsRNA is internalized, leading to the activation of dsRNA binding proteins: dsRNA dependent protein kinase (PKR), TLR3 receptor (TLR3), retinoic acid-inducible gene I (RIG-1) and melanoma differentiation-associated gene 5 (MDA5). The simultaneous activation of these signaling proteins leads to the rapid demise of the targeted cell and to a strong by-stander effect executed by the cytokines secreted by the targeted cell, and the immune cells converging to the cell that has been targeted. The by-stander effects lead to the destruction of neighboring tumor cells not targeted themselves by the PolyI:C armed vector. Normal cells, being more robust than tumor cells, survive. The strategy has a few advantages: (1) recruitment of the immune system is localized to the tumor, avoiding general pro-inflammatory effects that complicate other immune therapies. (2) It is fast acting such that treatment can be short, leading to rapid tumor eradication with minimal immunotoxic side effects. (3) The by-stander effects lead to eradication of tumor cells not harboring the target, a situation typical for the heterogeneous human tumors. (4) The multiplicity of pro-death signaling pathways elicited by PolyI:C, minimizes the chances for the emergence of resistance. In the lecture we shall focus on EGFR as the target since it is overexpressed in many tumors and on which we have accumulated strong pre-clinical data. In principle, the strategy can be implemented to other tumors that over-express a protein that can be internalized by a ligand, where the ligand can be a small molecule, a single chain antibody and an affibody.

Monday 9 July 2012

17:30–19:15

Symposium

Angiogenesis

Mechanisms of Resistance to Anti-VEGF Treatment – New Therapeutic Strategies

A. Harris1, 1John Radcliffe Hospital, Weatherall Institute of Molecular Medicine, Department of Oncology, United Kingdom

Anti-VEGF therapy is approved for a wide range of tumour types, and mechanisms of resistance to these treatments have been identified. However, the effect of these drugs varies from tumour type to tumour type for any one agent, and whether an agent active in one tumour type is active in another is variable. This variability is not currently explained. However, the vascular bed of every organ and tissue differs substantially from each other and a far better understanding of a molecular basis for this is necessary. Recent evidence indicates that tumour endothelium may originate from tumours themselves, which could explain some of the vascular heterogeneity.

We have investigated the variability of primary breast cancer in its response to Bevacizumab and conducted a window-of-opportunity study which revealed that there is great heterogeneity in response, with some patients showing progression during treatment, yet with others showing central necrosis developing within 2 weeks similar to vascular targeting.

Investigation of the pathways modified, using serial biopsies and exon arrays showed marked changes in metabolic pathways are induced within 2 weeks of a single dose of Bevacizumab at 15mg/kg. This includes induction of HIF1, decrease in proliferation, an increase in carbonic anhydrase 9 [CA9] expression and increase of PD1K and 3. Each of these observations therefore provides a new potential approach to synergise with anti-angiogenic therapy to enhance cell death and survival of patients. Here we show that there are no effect in response to DOVE like 4 generates resistance. This has been validated in retrospective analysis of a randomised trial.

Cancer has been investigated by using inducible CAS knockdown constructs and this demonstrates that inhibiting this key enzyme involved with regulation can substantially synergise with versus MAB therapy and we are currently developing small marker inhibitors for this that can also be used to image tumours. The induction of PD1 kinase also suggests changes in metabolism by mitochondria could be an important adaptation and this could be a subject for further investigation.

Recently polymorphisms in VEGFR1 have also been implicated in resistance. Overall there therefore is a strong likelihood that combination therapies exploiting the synthetic morphality of hypoxia plus drugs that block survival pathways will substantially increase the effectiveness of both therapies.


Anti-angiogenesis: Novel Strategies

No abstract received.

Proffered Paper: The Pro-angiogenic Phenotype of Natural Killer Cells Infiltrating Squamous Cell Carcinoma Lung Cancer

A. Bruno1, C. Focacott1, A. Pagani2, A. Imperatori3, A.R. Cantelmo1, C. Spinelli4, G. Ferlisi5, L. Martinelli1, A. Ablin1, D.M. Noonan1, 1Casa di Cura Multimedica – IRCSS, Oncologic Research, Milan, Italy, 2University of Insufuria, Biotechnologies and Life Sciences, Varese, Italy, 3University of Insufuria, Center for Thoracic Surgery and Department of Surgical and Morphological Sciences, Varese, Italy, 4University of Insufuria, Department of Human Pathology, Messina, Italy, 5University of Insufuria, Biotechnologies and Life Science, Varese, Italy

Introduction: The tumor microenvironment has come to light as a key player in carcinogenesis and progression. Tumors affect many host cell types, in particular immune cells. The immune system appears to select particularly fit tumor cells in the process of immuno-editing, while the tumor cells influence the polarization of immune cells towards phenotypes that favor tumor growth and vascularization. Here we investigated the subset distribution of tumor infiltrating natural killer (NK) cells, focusing on angiogenesis-associated cytokines production from clinical samples.

Material and Methods: Peripheral blood, adjacent normal and tumor tissues of patients with non-small cell lung cancer (NSCLC), as well as samples from non-oncologic patients, were collected and rapidly mechanically and enzymatically dispersed to obtain single cell suspensions. NK phenotype, subset distribution and functional characterization was performed by flow cytometry (FC) analyses for surface markers (CD3, CD56, CD16) and intracellular staining for VEGF, PI GF, IL-8 and INFg production. The ability of NK cells to induce angiogenesis was also assessed by endothelial chemotaxis and morphogenesis assays. Further, we evaluated the role of TGFb, a potent immune-suppressive tumor microenvironment-derived cytokine, in polarizing NKS towards a pro-angiogenic subset in terms of phenotype and function.

Results and Discussion: In NSCLC samples, the CD56+CD16- NK phenotype was associated with cytokine production, and predominated in the tumor samples, while the CD56dimCD16+ cytokine phenotype dominated in the adjacent normal tissues and in non-oncologic lung samples. The CD3- CD56+CD16-NK cells from both ADK and SQK, when stimulated in vitro, produce factors associated with angiogenic activities (VEGF, PI GF, IL-8) and are able to induce endothelial cell recruitment and morphogenesis, particularly evident for Squamous Cell Carcinomas (SQK) and absent from NK cells from non-oncologic patients. Exposure of naïve NK to TGFb in vitro polarized NKS to the CD56+CD16- subset and induced VEGF and PI GF production, suggesting a key role in promoting tumor angiogenesis.

Conclusion: Our data suggest that even NK cells are polarized toward an angiogenic phenotype in cancer, in particular for squamous NSCLC with a significant systemic effect on NK cells. NKS appear to participate in tumor neovascularization and could represent a marker for disease progression, angiogenesis and response to therapies in some tumor subsets.
Targeting Multiple Endothelial Growth Factor Pathways
K. Altaha1, 2, 3, C. Koufaris1, 2, 3

Solid tumors require blood vessels for growth and dissemination, and use lymphatic vessels as additional conduits for metastatic spread. The identification of growth factor receptor pathways regulating angiogenesis has led to clinical approval of the first antiangiogenic molecules targeted against the vascular endothelial growth factor (VEGF)-VEGFR-2 pathway. However, in many cases resistance to anti-VEGF-VEGFR therapy occurs, and thus far the clinical benefit has been limited to only modest improvements in overall survival. Therefore, novel treatment modalities are required. We will discuss the other members of the VEGF-VEGFR family as well as the angiopoietin growth factors and their Tie receptors as targets for the inhibition of tumor angiogenesis, lymphangiogenesis and metastasis.

Monday 9 July 2012
17:30–19:15
Symposium
Tumour Metabolism

Roles of p53 in the Control of Metabolic Pathways
K.H. Vouwen1, O. Maddocks2, C. Berker3, E. Cheung4, 5

The p53 protein is an important tumor suppressor that functions in a number of ways to prevent cancer development. Under conditions of severe or sustained stress, p53 can drive cell death and senescence, thereby removing the damaged and potentially transformed cell. However, recent evidence has also revealed an important role for p53 in promoting cell survival and modulating metabolism. In this context, we have recently found that p53 expression can help cells survive serine starvation. Serine starvation induces de novo serine synthesis by up-regulating the expression of enzymes in the serine synthesis pathway, causing the diversion of glycolytic intermediates and disruption of glycolysis. Interestingly, p53 is not necessary for the activation of the serine synthesis pathway, but seems to be required to allow cells to undergo this metabolic adaptation. The survival and adaptive activities of p53 could contribute to tumor suppression by helping cells to prevent or repair stress and damage but it is also possible that the inappropriate or deregulated expression of some of these activities of p53 may also support cancer progression. We have been investigating the activities of TIGAR, a p53-inducible protein that functions to protect cells from cell death. TIGAR can act as a fructose-2,6-bisphosphatase, driving the pentose phosphate pathway (PPP), promoting NADPH production to restore reduced glutathione and protecting the cell from ROS-associated apoptosis and autophagy. We have recently found that TIGAR also functions under conditions of hypoxia to limit mitochondrial ROS through mechanisms that are independent of its fructose-2,6-bisphosphatase activity. We have generated a TIGAR null mouse and have shown that although TIGAR is not necessary for normal growth and development, absence of TIGAR prevents tissue regeneration in the adult intestine following IR-induced ablation. We are now investigating the potential role of TIGAR in supporting malignant development.

Proferred Paper: Regulation of Cellular Metabolism by Cancer Genes – Implications in Breast Cancer
A. Carracedo1, A. Lelaitert2, M. Bhassir3, K. Ito4, P. Pulgserver4, M. Haigis5, E. Maratos-Flier6, 7, 8, A. Richardson9, Z.T. Schafer2, P.P. Pandolfi4, 10

We have been investigating the activities of TIGAR, a p53-inducible protein that functions to protect cells from cell death. TIGAR can act as a fructose-2,6-bisphosphatase, driving the pentose phosphate pathway (PPP), promoting NADPH production to restore reduced glutathione and protecting the cell from ROS-associated apoptosis and autophagy. We have recently found that TIGAR also functions under conditions of hypoxia to limit mitochondrial ROS through mechanisms that are independent of its fructose-2,6-bisphosphatase activity. We have generated a TIGAR null mouse and have shown that although TIGAR is not necessary for normal growth and development, absence of TIGAR prevents tissue regeneration in the adult intestine following IR-induced ablation. We are now investigating the potential role of TIGAR in supporting malignant development.
Mass Spectrometric Strategies for Protein Biomarker Discovery and Validation

R. Aebersold1, 1ETH Zurich, Institute of Molecular Systems Biology, Zürich, Switzerland

A key barrier to the realization of personalized medicine for cancer is the identification of biomarkers. Of all types of biomarkers, plasma protein biomarkers are particularly attractive because they can be measured in easily accessible samples. Unfortunately, the search for plasma protein biomarkers has been highly challenging and met with surprisingly low level of success. Specifically, the comparison of plasma sample proteomes of control and disease affected individuals has to date not uncovered any new markers. On the backdrop of the emerging personal genome information and large scale cancer genome projects we have developed and applied a biomarker strategy that is driven by cancer genetic and genomic information. In a fast stage we use comparative genomic data to computationally predict which signaling systems might be perturbed in a particular type of cancer. We use targeted proteomic measurements on human tissue samples or tissue samples from suitable mouse models to experimentally validate these predictions, i.e. to determine which proteins are disregulated in the specific disease. We then use then the such validated perturbed molecular networks to select proteins that are likely to be secreted or otherwise released into plasma and quantify these proteins in sets of plasma samples by selected reaction monitoring, a highly sensitive targeted mass spectrometry technique.

In this presentation we will discuss this novel biomarker strategy, its present status and expected directions. A case study on PTEN dependent prostate cancer will illustrate the concept.

The A-Z of Clinical Trials – Clinical Trial Design Incorporating Efficacy, Translational and Biomarker Endpoints

E.A. Eisenhauer*, 1NCIC Clinical Trials Group, Cancer Research Institute Queen's University, Kingston, Canada

Clinical trial design and endpoints in the era of targeted therapeutics have been the subjects of considerable debate. The key question in this discussion is how best to undertake clinical development of new agents to assess, in parallel, proof of mechanism, efficacy and also identify predictive biomarkers for personalized (or precision) treatment decisions.

Phase I trials are first-in-human evaluations that aim to identify the recommended dose of the agent for further study by evaluating adverse effects (toxicity) pharmacokinetics (PK) and effects of drug on target (PD) in tissue of patients. Their design and successful conduct is dependent on preclinical data available on these same measures. Phase I trials enroll small cohorts of patients at escalating doses of the new agent and assess toxicity, PK and PD at each dose level. If tumor tissue itself is to be sampled for PD, then often that is undertaken only at highest doses (or at recommended phase II dose). Clearly a robust assay is required for PD assessment, particularly if this is to be instrumental in determining the recommended dose or go/no go development decisions for the compound. A variety of dose escalation schemes are available and the clinical research community continues to ponder the best endpoints upon which to define dose recommendation (toxicity, PK or PD or some combination).

The role of phase II trials is to ’screen’ new agents for efficacy. There is controversy about whether population enrichment for potential predictive biomarkers should begin in phase II or whether this stage of development should allow a broad range of patient entry in order to refine hypotheses about predictive biomarkers for later validation. Clearly, the answer to this depends, in part, on how much is known about the putative target/biomarker prior to phase II and the availability of an assay to measure it. In addition, there is debate about whether tumor regression or other endpoints are most useful/appropriate in such screening trials. While some view tumor regression in non-randomized trials as a valid screening approach, others argue for the use of progression or stable disease to assess anti-tumor effects. These latter endpoints require randomized designs and larger sample sizes.

Phase III trials are large studies that are definitive tests of efficacy of new agents (given alone or in combination). These are randomized comparative studies with a reference arm comprised of standard of care. Increasingly biomarker measures are incorporated into phase III trials either to validate putative biomarkers acquired during phase II or to inform biomarker discovery work if predictive/selection marker remain unknown. Occasionally, biomarker data from phase II or preclinical data is sufficiently robust that the biomarker-defined population alone is studied in phase III. However, this has only rarely been undertaken for the targeted agents studied in phase III in the past decade. Phase III trials are powered to detect a postulated meaningful difference in a major efficacy endpoint – generally overall survival (OS) in metastatic disease studies, though there has been a trend to utilizing progression free survival (PFS) in recent years, particularly for agents with targeted molecular mechanisms. This is a somewhat worrying trend since it is unclear if prolongation of PFS of a few weeks or months is meaningful on its own, without overall survival or quality of life gains. The optimal primary endpoint is one of the most widely debated issues in phase III design. Furthermore, there has been a growing interest in using statistical designs that are “adaptive”, i.e. where the sample size, trial arms or selection biomarker are altered during the course of the study based on emerging study results.

Examples of new drugs and trial design will be used to illustrate these topics throughout the lecture.

Cancer Immunotherapy: Strategies for Enhancing its Activity in Cancer Patients

F. Belardelli1, F. Moretti1, 1Istituto Superiore di Sanità, Department of Hematology Oncology and Molecular Medicine, Rome, Italy

The origin of the cancer immunotherapy dates back almost 120 years, when William B. Coley, a New York surgeon, described the antitumor effect of the first “biological response modifier”, represented by a mixture of bacterial products. Since then, an impressive progress has been made not only in our understanding of the cellular and molecular mechanisms responsible for a protective antitumor immune response, but also in the clinical development of well defined new molecules (especially certain cytokines and monoclonal antibodies). Likewise, new immunotherapy strategies, including cell therapies and cancer vaccines, alone or in combination with conventional anticancer treatments, have been tested in clinical trials. In particular, cancer vaccines represent an attractive strategy for achieving a long-term antitumor immune response for several human malignancies, but their clinical development has been characterized by alternate cycles of optimism and discouragement. Tumor cells express a variety of self tumor-associated antigens (TAA) recognised by the host’s immune system and multiple modalities for using them have been tested in order to induce a clinically relevant antitumor immune response in cancer patients. A major current challenge is the identification of new and effective strategies for enhancing the efficacy of cancer vaccines. One approach consists in the use of new adjuvants and biological response modifiers (including certain cytokines) capable of breaking tolerance against TAA, thus enhancing the immunological and clinical response to cancer vaccines. Over the last 15 years, different types of dendritic cells (DC) have been used as autologous cellular adjuvants for the clinical development of therapeutic vaccines with variable results. A critical overview of the available clinical data and the recent advance in tumor cell immunology suggests the interest of exploring novel types of well characterized DC-based therapies, alone or in combination with additional interventions, and to identify reliable biomarkers for predicting or monitoring patients’ response. Since the early clinical studies of cancer immunotherapy, the question arose as to whether it...
was possible to combine it with standard treatments such as chemotherapy. The answer, now, is past history. The combined use of immunotherapy and chemotherapy is not only possible but, in certain cases, depending on the drug, the dose and the combination modalities. In order to find the best synergisms between the two treatments and to turn weak immunotherapeutic interventions into potent anticancer instruments, it is mandatory to understand the complex mechanisms responsible for the positive interactions between chemotherapy and immunotherapy. The results of an ensemble of studies carried out in our laboratory as well as by others in both mouse models and patients suggest some novel mechanisms that could be exploited for enhancing the clinical response to cancer immunotherapy.

Meet the Expert

**Molecular Diagnostics in Breast Cancer – Getting Beyond ER, PR, HER2 and Proliferation**

J. Reis-Filho, 1 Institute of Cancer Research, Breakthrough Breast Cancer Research Centre, London, United Kingdom

Breast cancer comprises a collection of different diseases, which have different histological features, molecular profiles, clinical behaviour and response to therapies. In the last 10 years, several lines of evidence, largely stemming from high throughput molecular profiling studies, have demonstrated that oestrogen receptor (ER)-positive and ER-negative breast cancers are fundamentally different diseases. The excitement with novel technologies, in particular gene expression profiling, led to the promise that the potentials of personalised medicine could be realised in an expeditious manner. It should be noted, however, that over a decade after the introduction of microarray-based gene expression profiling, molecular diagnostic methods for the management of breast cancer patients are still restricted to the assessment of ER, progesterone receptor (PR) and HER2 status, and the levels of tumour cell proliferation. Arguably, the main contributions of gene expression profiling were in the unravelling of the molecular heterogeneity of breast cancers, the identification of the differences between ER-positive and ER-negative breast cancers, and the importance of proliferation as one of the main determinants of the outcome of patients with ER-positive disease. Furthermore, this type of approach has led to the implementation of multiparameter predictors of outcome, which are now being used in clinical decision-making, based on either clinicopathological and/or transcriptomic data. It is now possible to define good prognosis ER-positive breast cancer patients with great accuracy, to point out the limitations of research endeavours to refine the prognostication of these patients even further may prove futile. On the other hand, several questions that are germane to the realisation of the potentials of personalised medicine remain to be answered. It is still unclear as to how breast cancer should be subtyped from a molecular standpoint; prediction of response to specific therapeutic agents is still imprecise; and methods to account for the intratumour phenotypic and genetic heterogeneity found in breast cancers remain to be fully developed. In this talk, the state of the art in molecular diagnostics of breast cancer patients, the promise of new technologies, including massively parallel sequencing, and the challenges that lie ahead will be discussed.

**Biobanking**

P. Kriegman, 1 M.M. Morente, 2 J.A. López-Guerrero, 3 M. Grazia Daidone, 4 T. Söderström, 5 J. Thompson, 6 J. Hall, 7 M. Mainuma, 8 A. Broeks, 9 V.P. Collins 10 1 Erasmus MC, Department of Pathology, Rotterdam, The Netherlands, 2 Fundacion Centro Nacional de Investigaciones, Molecular Pathology, Valencia, Spain, 3 IFCCS Istituto Nazionale Dei Tumori, Department of Pathology, Milan, Italy, 4 Karolinska Institute, Department of Pathology, Stockholm, Sweden, 5 Karolinska University Hospital, Department of Pathology, Stockholm, Sweden, 6 EORTC, Translational Research and Imaging, Brussels, Belgium, 7 IARC, Laboratory Services and Bio Bank Group, Lyon, France, 8 NKI, Pathology, Amsterdam, The Netherlands, 9 University of Cambridge, Pathology, Cambridge, United Kingdom

Openly sharing human samples collected in a standardized way between hospital integrated biorepositories and their research groups would enable multi center translational cancer research having a high statistical significance impact with possible direct consequences on innovation of patient care. Moreover, the speed in which these experiments could be performed would be unmatched to the standards of today. Sharing samples is seen as a no go area for most investigators (collection stakeholders), afraid to lose on their institutional and departmental investment of resources and it is seen as difficult because institutional bias can disrupt the expected outcome and there are many regulatory issues involved when exchanging samples. Biobanks have tried to set up networks enabling scientists to find samples they need. However, the enthusiasm to upload sample data is not always shared happily without knowing benefits in advance.
New Targeted Agents in Development for Cancer Treatment

M. Pajic1, D.K. Chang1, K.S. Kassahn2, J.Wu1, M. Cowley1, N. Waddell2, J.Soria1, C.P. Massard1.

1Program, Sydney NSW, Australia, 2University of Queensland, Institute for treatment of cancers.

Recent developments in high-throughput technologies for gene sequencing, showed overall survival improvement in melanoma and prostate cancer. Moreover, novel immunotherapeutic strategies (anti-CTLA4 and vaccine) and inhibitors targeting FGFR1, MET, MEK, PI3K, etc, are in clinical testing. A small portion of patient corresponding to 5% of adenocarcinomas, and this inhibitor, was tested in patients with CRPC pre-treated with docetaxel. These new anti-cancer drug developments are an enormously long, complex and costly process, leading to many inequalities among patients for access to innovation, depending mostly of geographic origins and socio-economic levels. This issue becomes even more complex when about 900 new targeted agents are under development, any of them expected to be of therapeutic interest in only a small still undetermined subset of patients. Furthermore, examples are now accumulating showing that the most promising of those drugs will have a long-term effect only when given in combinations, in attempts to target efficiently cellular key pathways at several levels (or to target several parallel pathways) in order to avoid subclonal selection of additional molecular events leading to tumor resistance (anti-B-RAF in melanomas, resistance to Herceptin in HER-2 positive breast cancers). Such data illustrate how important would be to initiate biology-driven multiple drug combinations early in drug development, overcoming the fact that relevant drugs are commonly developed by different currently competing firms.

Preclinical Models of Pancreatic Cancer

M. Paic1, D.K. Chang1, K.S. Kassahn2, J.Wu1, M. Cowley1, N. Waddell2, A. Johns1, Australian Pancreatic Cancer Genome Initiative2, S.M. Grimmond2, A.V. Blankin1.

1Garvan Institute of Medical Research, Cancer Research Program, Sydney NSW, Australia, 2University of Queensland, Institute for Molecular Biosciences, St Lucia QLD, Australia, 3www.garvan.org.au/apgi. NSW, Australia

Background: Pancreatic cancer (PC) is the fourth leading cause of cancer death in Western societies with an overall 5-year survival rate of less than 5%. There are few therapeutic options for patients with PC, and new insights into the pathogenesis of this lethal disease are urgently needed.

Methods: Recent advances in nucleic acid sequencing technology have made it feasible to rapidly, and exhaustively sequence an entire genome, including comparative genomic hybridization. They have revealed many known molecular abnormalities and described some new alterations with clinical interest. Several prospective trials are ongoing to treat patients with advanced cancer according to the existence of oncogenic driver events (mutations/translocations etc.), and to demonstrate that the development of personalized medicine with a focus on novel targeted therapies will challenge the one-size-fits-all approach to the treatment of cancers.

Results: Our cohort of primary xenografts generated directly from resected human PC is extensively characterised (genome, transcriptome, epigenome and phosphoproteome) as part of the ICGC, and histologically recapitulates the human disease, including a significant stromal component. Since these xenografts retain the genomic signature of primary PC, are generated from individual patients, and are renewable, multiple treatments can be simultaneously examined, providing the opportunity to test personalised medicine strategies, and facilitate novel therapeutic development linked with companion biomarker discovery that are currently intractable in a clinical trial setting. In an interim integrative analysis of DNA copy number and whole genome aberrations in a 100 pancreatic tumors, we have identified several molecular phenotypes that have now been examined in the preclinical models and have the potential to inform decisions on optimal treatment strategies for individual tumours.

Conclusion: We demonstrate that integrative analysis of multidimensional data encompassing genomic sequence, copy number variation, gene expression and methylation can be utilized to develop new therapeutic strategies that demonstrate efficacy in model systems. This approach may be used to define biologically and clinically relevant cancer phenotypes.
expression of stem cell factors, as demonstrated for the polyc明白了 repression Bmi1. We propose that ZEB1 links EMT-activation and stemness-maintenance by suppressing stemness-inhibiting microRNAs and thereby is a promoter of mobile, migrating cancer stem cells. Notably, these cells also acquired a drug-resistance phenotype. Thus, targeting the ZEB1–miR-200 feedback loop might be a promising treatment option for fatal tumors, such as pancreatic cancer.

Regulation of Epithelial Plasticity and Metastasis of Breast Cancer by Lysyl Oxidase-like 2 (LOXL2)

A. Cano1, G. Moreno-Bueno1, 1Universidad Autonoma de Madrid Instituto de Investigaciones Biomedicas “Alberto Sols” CSIC-UAM, Biochemistry/ Cancer Biology, Madrid, Spain

Background: Lysyl oxidase-like 2 (LOXL2), a member of the lysyl oxidase (LOX) family, has been previously shown to interact with Snail1 promoting Snail1 stabilization and epithelial-mesenchymal transition (EMT) [1]. Our previous studies identified LOXL2 as a poor prognosis marker in larynx squamous cell carcinomas inducing malignant transformation by both Snail1-dependent and -independent mechanisms and revealed that LOXL2 overexpression is associated to poor prognosis of N0 breast carcinomas [2]. In the present study we have investigated further the implication of LOXL2 in breast carcinoma tumors.

Material and Methods: A combination of transcriptomics and immunohistochemistry analyses for LOXL2 in a series of grade 3 IDC breast carcinomas (n = 195) have been carried out. Breast tumors samples were from the archives of the Pathology Departments of Hospital La Paz, Madrid, and MD Anderson Cancer Center Madrid. LOXL2 expression analysis were performed at mRNA and protein level in a collection of human breast carcinoma cell lines, representative of different breast tumor subtypes (luminal; ErbB2+; and triple negative: ER−/PR−/ErbB2−). Loss of function studies by shRNA have been carried out in basal carcinoma cells (MDA-MB231; BT549) and non-tumorigenic breast basal cells (HBL100) followed by phenotypic and molecular characterization and studies of tumorigenity and metastasis.

Results: Expression profiling studies of breast carcinomas grade 3 IDC tumors (n = 58) identify a metastatic signature for basal-like breast tumors (BBS: Basal-like Breast Signature), a subtype of highly aggressive triple negative breast tumors. Basal-like breast carcinomas have been previously described as prone to suffer epithelial-mesenchymal transition (EMT) [3,4]. Interestingly, LOXL2 expression was confirmed to be restricted to basal-like tumors by qRT-PCR. Immunohistochemical analyses in an extended series of grade 3 IDC (n = 195) showed that increased LOXL2 expression with an intense cytoplasmic/perinuclear pattern significantly correlated with the subgroup of metastatic basal-like breast tumors. In agreement with those findings, LOXL2 is expressed with the intense perinuclear pattern in breast carcinoma cell lines of basal-like Bimesenchymal phenotype and in non-tumorigenic basal cells, but it is completely absent in carcinoma cells of the luminal and ErbB2 subtypes. Silencing of LOXL2 in basal-like breast cells by shRNA induces a dramatic reversion of the phenotype leading to mesenchymal-epithelial transition (MET), associated to reduced cell motility and invasion of basal-like cells. Furthermore, LOXL2 knockdown reduced the tumor growth potential and completely suppressed the lung metastatic colonization of highly aggressive MDA-MB-231 cells. Remarkably, the MET-like process induced by LOXL2 knockdown in basal-like cells is independent of E-cadherin and Snail1 modulation, but involves the upregulation and reorganization of tight junctions and cell polarity complexes at the apico-lateral membrane and downregulation of FAK activation. Interestingly, LOXL2 is able to repress tight junction (Claudin1) and cell polarity components (Lgl2) at transcriptional level by a mechanism independent of Snail1 and LOXL2 catalytic activity [5].

Conclusions: These results indicate that intracellular LOXL2 is required for metastatic dissemination of basal-like breast tumors. The newly uncovered role of LOXL2 in the negative modulation of cell polarity and the epithelial phenotype may unveil new targets for anti-metastatic therapies of highly aggressive basal-like tumors.

Reference(s)

The Basics of Melanoma Cell Signalling

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**Background:** The protein kinase BRAF is mutated in about 50% of human melanomas. Drugs such as vemurafenib that inhibit BRAF can mediate impressive responses in melanoma patients whose tumours express the mutant form of BRAF. However, about a third of these patients develop keratoacanthomas or squamous cell carcinomas (SCC).

**Material and Methods:** We have used a combination of molecular biology, cell biology and murine models to investigate responses to BRAF inhibitors in vitro and in vivo.

**Results:** We show that although BRAF inhibitors inhibit cell signalling in cells that express oncogenic BRAF, paradoxically they activate cell signalling in cells that express oncogenic RAS. This is because in the presence of oncogenic RAS, BRAF inhibitors drive the formation of homodimers and heterodimers between BRAF and the closely related protein CRAF, leading to hyper-activation of RAF and consequently elevated signalling. Notably, about 60% of the non-melanoma skin lesions that develop in patients treated with BRAF inhibitors carry mutations in RAS and we used a two-stage skin carcinogenesis model of SCC to show that BRAF inhibitors are not tumour promoters in their own right; rather they act to accelerate the induction of these non-melanoma skin cancers in already susceptible individuals through the paradoxical activation of the RAF/MEK/ERK signalling pathway. Notably, the induction of these lesions is blocked by co-administration of BRAF and MEK inhibitors. Paradoxical reactivation of the MEK/ERK pathway also underlies the mechanisms of resistance to BRAF inhibitors in melanoma patients.

**Conclusions:** Our data reveal the complex nature of RAF/MEK/ERK signalling in cancer cells. They explain the mechanisms by which the side effects are mediated and explain some of the mechanisms of resistance. Furthermore, our studies demonstrate how deeper understanding of this pathway has led to improved treatment options for melanoma patients.

**Treating Metastatic Melanoma With the BRAF Inhibitor Vemurafenib and Mechanisms of Resistance**

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Activating mutations in kinases of the MAPK pathway are known to drive proliferation in the majority of melanomas. Mutations in BRF are found in 40–60% of melanomas. Vemurafenib and dabrafenib are inhibitors of mutated BRAF that have been shown to induce responses in the majority of patients treated with confirmed response rates of 50%. Recently, Phase III trials comparing vemurafenib with dacarbazine showed a significant improvement in overall survival (hazard ratio 0.44) and progression-free survival (hazard ratio 0.26). As a result, vemurafenib was approved by the US FDA and the EMA for use in metastatic melanoma patients harboring a BRAF mutation. A Phase III trial comparing dabrafenib with dacarbazine has been completed and the results are awaited. Both of these drugs have been shown to cross the blood-brain barrier and have significant activity on brain metastases. Because the median progression-free survival is only 6–7 months, significant effort has been made in investigating mechanisms of resistance. In all resistant tumors investigated to date, the MAPK pathway is re-activated. Two confirmed mechanisms reported have been re-activation of the MAPK pathway through activating mutated BRAF splice variants and NRAS mutations. There is some evidence that in other cases there is activation of receptor tyrosine kinases. There are several challenges for the field going forward. Among these are preventing resistance to BRAF inhibition, induction of durable complete responses, and developing effective strategies for interrupting the MAPK pathway in melanomas not having a BRAF or KIT mutation.

Proffered Paper: The Importance of Glycolysis for the Response and Resistance of BRAFV600E Human Melanoma Cells to Vemurafenib

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**Background:** Cancer cells have unique metabolic requirements compared to normal proliferating cells. In particular, dependency on glucose for ATP production and macromolecular biosynthesis has been described in almost all cancers. We propose that oncogenes drive a dependency on glucose metabolism that is vital for responses and resistance to oncogene-targeted therapies such as vemurafenib (vem), a highly selective BRAF inhibitor approved for the therapy of BRAFV600E melanoma. Phase III clinical trials of vem have reported striking response rates in excess of 50% in patients diagnosed with BRAFV600E metastatic melanoma. However, vem resistance develops clinically after 6–8 months and is a major focus for further clinical development of BRAF inhibitors.

**Methods:** The effect of vem on glycolysis was examined in detail in a panel of human melanoma cell lines, including BRAFV600E and BRAFWT cell lines and a series of cell lines with acquired resistance to vem.

**Results:** Vem suppressed glucose uptake and lactate/ATP production in BRAFV600E human melanoma cells and a strong positive correlation existed between vem proliferation IC50 and the extent of inhibition of glucose uptake ($r^2=0.73$, $p<0.001$). Notably, inhibition of glucose uptake was independent of vem-induced cell cycle arrest/apoptosis, indicating that ERK/MAPK signalling directly regulates glycolysis. Accordingly, vem treatment decreased glucose transporter-1 (GLUT1) and GLUT3 mRNA and membrane protein expression in BRAFV600E melanoma cells and inhibited expression of hypoxia inducible factor-1α (HIF1α), a major regulator of GLUT1/3 expression. Furthermore, HIF1α stabilisation during hypoxia significantly reduced sensitivity to vem in BRAFV600E cells. Thus, suppression of HIF1α stability by vem may inhibit GLUT1/3 expression. This is likely to underlie the inhibition of glycolysis by vem and may be important for vem responses in BRAFV600E melanoma cells.

**Conclusions:** BRAF inhibition suppresses glucose metabolism in BRAFV600E human melanoma cells and this involves HIF1α-mediated regulation of GLUT1/3 expression. Furthermore, the reversal of vem resistance by DCA strongly supports development of drug combinations that incorporate metabolic inhibitors such as DCA, and targeted therapeutics such as vem, for the treatment of oncogene-driven cancer.
Semaphorin 7A is Upregulated in Human Breast Cancer and Promotes Epithelial to Mesenchymal Transition in the Breast Epithelia

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Introduction: Metastasis is one of the major causes of cancer deaths. Our laboratory is studying the role of the immune system in enhancing metastasis. We show that axonal guidance molecule Semaphorin 7A is expressed by both tumor cells and the immune cells of mammary tumor bearing mice. We are the first to study Sema7A in the context of cancers.

Material and Method: Tumour specimens from breast cancer patients that presented tumors greater than 1.5 cm were assayed for Sema7A gene expression by Q-PCR. For in vivo studies, we implanted BALB/c mice with 4T1-LUC-2 control cells expressing Sema7A or 4T1-LUC-2 cells that silenced this gene. Both cell lines were assayed for genes related to epithelial to mesenchymal transition and metastasis using gene arrays. The compliance of these cells were measured using atomic force microscopy.

Results and Discussion: We found that Sema7A is up-regulated in the tumor tissues of breast cancer patients but expression is minimal in the adjacent normal tissue. We show that differential expression of Sema7A in breast cancer cells lines enhances a mesenchymal/stem-like phenotype and affects the compliance measurements of breast cancer cells. Furthermore, implantation of breast tumor cell lines with altered Sema7A expression in BALB/c mice affected tumor growth, metastasis and survival. Cells that were silenced for Sema7A expression showed a decreased mesenchymal phenotype, had lower metastatic and angiogenic potential.

Conclusion: Sema7A could prove to be a novel therapeutic target to limit tumor growth and metastasis not only for breast cancer but for other solid tumors as well.

Defining the Involvement of the E6AP-PML Axis in Prostate Cancer

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Introduction: Type II E6AP-PML axis is critical for the formation of distinct nuclear structures called PML-nuclear bodies (PML-NBs), which are important for PML activity. We have previously discovered that the E3 ligase E6-associated protein (E6AP) is a major regulator of PML protein stability and the formation of the PML-NBs. PML has been extensively studied in human cancers and its expression has been found to be frequently down-regulated or lost. In prostate cancers, the loss of PML is also associated with the development and progression of invasive prostate cancer. Interestingly, the regulation of PML occurs at the protein level, which prompted us to hypothesize that deregulation of E6AP may lead to the down-regulation of PML, thereby playing a role in the development and progression of human prostate cancer.

Results and Discussion: We have screened prostate cancer samples for the expression of E6AP and PML, and correlated this data with survival following radical prostatectomy. We found that patients with high E6AP and low PML expression levels have the poorest survival rates. To test our hypothesis we examined the correlation between E6AP and PML expression in a panel of prostate cancer cell lines. We studied the effect of E6AP on PML expression by down-regulation of E6AP using inducible shRNA in these cell lines. The down-regulation of E6AP successfully restored PML expression and enhanced the formation of PML-NBs. We have also observed a significant reduction in cell numbers when E6AP was down-regulated. The mechanism underlying the effect of E6AP down-regulation on cell proliferation will be discussed.

Conclusion: Our study reveals a novel role for the involvement of E6AP-PML axis in prostate cancer.
In Vitro Evaluation of Antioxidant and Antitumoral Activities of Marine Algae Gelidium Sesquipedale and Fucus Spiralis

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Material and Methods: significant cytotoxic activity

Results: level in human platelets using flow cytometry. MTT assay against the target cells: human cervix carcinoma (HeLa), and alga Gelidium sesquipedale (Clemente) Thuret (Rhodophyta) and brown alga Fucus spiralis (Phaeophyta). Material and Methods: Dichloromethane-methanol (50:50) extracts of red alga Gelidium sesquipedale and brown alga Fucus spiralis found on the Atlantic coast of Morocco were screened for the studies on their antitumoral action. In vitro cytotoxicity of the extracts was tested by MTT assay against the target cells: human cervix carcinoma (HeLa), and chronic myelogenous leukemia (K562). H2DCFDA (dichlorodihydrofluorescein diacetate)-cell-permeable indicator was used to measure intracellular ROS level in human platelets using flow cytometry.

Results: The obtained results showed that both studied extracts expressed significant cytotoxic activity in vitro toward malignant HeLa, and K562 cell lines. The IC50 values in the MTT assay for Gelidium sesquipedale were 38.03 ± 2.62 (µg/ml) for HeLa and 18.41 ± 1.86 (µg/ml) for K562 cell lines. C50 values for Fucus spiralis were ranged from 36.70 ± 1.65 (µg/ml) for HeLa, and 17.34 ± 1.12 (µg/ml) against K562 line. Also, the treatment of human platelets by the extracts resulted in reduction of intracellular ROS level induced by the treatment of human platelets with arachidonic acid. Conclusions: Results obtained indicate the potential of these extracts for the antitumor action, making them particularly interesting for further in vitro and in vivo investigations.

106 Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR) Reveals Increased Cholesterol Ester Content in Drug Resistant Laryngeal Carcinoma Cells

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Background: Infrared spectroscopy is developing as a new and more holistic approach to studying drug resistance, yielding a unique fingerprint of all molecules present in the cell. In our laboratory we have developed a human laryngeal carcinoma (HEP-2) subtype resistant to carboplatin (7T) and cross resistant to the natural compound curcumin. In our previous work we have reported that following treatment with curcumin, 7T cells exhibited reduced accumulation of curcumin, induction of reactive oxidative species (ROS) and Hsp70 expression, as well as diminished lipid peroxidation, oxidative and basic DNA damage, G2/M phase arrest, and the induction of apoptosis, as compared to parental HEP2 cells. In order to shed more light on the molecular mechanism of curcumin resistance in 7T cells, we used FTIR-ATR spectroscopy.

Materials and Methods: HEP-2 and 7T cells were independently grown and samples were collected. Times. The fingerprint spectra of both, HEP-2 and 7T exponentially growing cells were recorded with the FTIR Perkin-Elmer GX spectrometer. 5 x 10^4 cells in 20 µL of cell suspension were analyzed in three replicates. The data analysis was performed using the FTIR-ATR spectral analysis. Cell spectra were obtained in the 4000-4800 cm^-1 region, 200 scans and at a resolution with 4 cm^-1 at room temperature. The data analysis was carried out using the MATLAB program with two add-ons, PL3_Toolbox and KineticToolbox.

Results and Discussion: By comparing the spectra from parental HEP-2 and resistant 7T subline, we found that the most interesting difference was the increase in cholesterol ester content recorded in resistant 7T cells. According to the literature, cholesterol esters are localized in lipid droplets inside cells. Using the Oil Red O dye we confirmed the existence of lipid droplets in resistant 7T cells and also that their quantity and presence in the cell culture is serum dependent. However, almost none of these properties were found in HEP-2 cells.

Conclusion: We can conclude that in addition to previous determined molecular mechanisms involved in resistance of 7T cells to curcumin, these cells exhibit increased content of cholesterol esters suggesting an alteration in their cellular metabolism. In addition, our results clearly show the advantages of FTIR-ATR spectroscopy as a new technique in cellular or molecular biology research.

107 Clinical Value of Proliferation and Apoptosis Markers in Osteogenic Sarcoma Tumor Cells

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Background: To find out prognostic role of expression of apoptosis and proliferation in osteosarcoma. Materials and Methods: 46 patients to 30 ages have been examined, 30 – males and 16 – females. Full effect and partial effect were marked in these patients, but expression of genes mtp53, Bcl-2 and Ki-67 in tumor cells have been studied by the method of immunohistochemistry with monoclonal antibodies (Novocastra). Visualization have been executed using of Streptavidin – HRP and DAB (Dakocytomation). Results: Organ-conserving operations have been performed in 16 patients (10 males, 6 females). Full effect and partial effect were marked in these patients, but expression of genes mtp53 and Ki-67 was low (1+) or absent. Expression of genes bcl-2 was also low (1+). Crippling operations have been performed in 20 males and 10 females with osteosarcoma, in 9 patients of them was detected metastasises further. Partial effect and stabilization of tumoral process was marked in all patients of this group, but the expression of genes mtp53 and Ki-67 was negative (0). In tissues metastasises further, expression of genes bcl-2 was moderate (2+) and high (3+). Conclusion: By study of the expression level of genes mtp53, Ki-67 and bcl-2 in tumor cells, we can use conducted therapy for effective prognosis in the choice of osteosarcoma sarcoma treatment.

108 The Deubiquitinase USP9X Suppresses Pancreatic Ductal Adenocarcinoma

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) remains a lethal malignancy despite tremendous progress in its molecular characterization. Indeed, the biological sequence of PDAC has been partially attributed to frequent and well characterized mutations in KRAS (80%), CDKN2A (90%), TP53 (70%) and SMAD4 (90%), and a plethora of lower frequency genetic events of uncertain significance. Recent genome-wide analyses have uncovered numerous additional somatic genetic alterations, although the functional relevance of most remains uncertain. Therefore, the identification of novel ‘drivers’ will allow a better understanding of the pathogenesis of this disease.

Material and Method: We have used Sleeping Beauty (SB) transposon-mediated insertional mutagenesis in a mouse model of pancreatic ductal preneoplasia to identify genes that cooperate with oncogenic Kras to accelerate tumorigenesis and promote progression. A cohort of 117 KCTSB13 (Kras<sup>−/−</sup>; Pdx1-cre; T2; Onc; Rosas26-LSL-SB13) mice was monitored for tumor development. KCTSB13 mice rapidly progressed and succumbed to invasive pancreatic neoplasms. A total of 198 distinct primary tumors and metastases were subjected to histological and molecular analysis. Spink1 or Spink4 PRs were performed to identify common insertion sites (CIIs) associated with candidate cancer genes.

Results and Discussion: Our screen has revealed new candidates and confirmed the importance of many genes and pathways previously implicated in human PDAC. Interestingly, the most commonly mutated gene was the X-linked deubiquitinase USP9x, which was inactivated in over 50% of the tumors. We found that loss of USP9x enhances transformation and protects pancreatic cancer cells from anoikis. Clinically, low USP9x protein and mRNA expression in PDAC correlates with poor survival following surgery, and USP9X levels are inversely associated with metastatic burden in advanced disease. Furthermore, chromatin modulation with trichostatin A or 5’aza-2’- deoxycytidine elevates USP9X expression in human PDAC cell lines to suggest a clinical approach for certain patients. The conditional deletion of USP9x correlated with Kras<sup>−/−</sup> to rapidly accelerate pancreatic tumorigenesis in mice, validating their genetic interaction.

Conclusion: We propose USP9X as a major new tumor suppressor gene with prognostic and therapeutic relevance in PDAC. Moreover, the identification
Tyrosine Kinase Receptors as Functional Markers and Therapeutical Targets of Glioblastoma Stem Cells

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Introduction: Glioblastoma (GB) is the most common and aggressive primary brain tumor, with an estimated median survival of 15 months after diagnosis. However, thanks to the recent identification of genetic lesions and transcriptional profiles, GB can be classified into subtypes (‘pro-neural’, ‘classical’ or ‘mesenchymal’), each endowed with distinct biological and prognostic features, and associated with new potential therapeutic targets. The proneural profile is characterized by mutations of IDH1/2 gene and alterations of the PDGF signaling pathway. Classical and mesenchymal subtypes are characterized by activating lesions of the EGFR gene, or by mutations/deletions of PTEN and NF1 genes, respectively. GB tumors are also known to be organized into a cellular hierarchy, led by a stem-like cell subpopulation that retains the tumorigenic potential, and gives rise to a heterogeneous, pseudodifferentiated bulk population. GB cell subpopulations display different sensitivity to therapies, and the stem-like subpopulation has been shown to possess high inherent radio- and chemoresistance: thus, GB stem cells are likely responsible for treatment failure and tumor recurrence. We undertook systematic characterization of tyrosine kinase receptor expression and function in GB stem cells, in order to find out new therapeutic targets.

Material and Methods: Neurospheres, i.e. cultures enriched in stem and progenitor cells, were isolated and propagated in vitro. Neurospheres underwent mutational and transcriptional profiling, immunophenotyping, analysis of proliferative and invasive abilities in vitro and tumorigenic potential in vivo.

Results: We found that neurospheres displayed the same mutational profile, and mirrored the same gene expression profile as the original tumors, and could thus be classified as ‘classical’, ‘mesenchymal’ or ‘pro-neural’. We then specifically associated the expression of EGFR to the ‘classical’, and MET and FGFR to the ‘mesenchymal’ or ‘pro-neural’ subtypes, and showed that these cells rely on these receptors for their biological activities.

Conclusions: We have shown that tyrosine kinase receptors are subtype-specific functional markers in glioblastoma, and can be considered as potential therapeutic targets.

EMT Followed in Three Dimensions in Vitro

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Introduction: The clinically important process of metastasis is difficult to model in vitro because it is dependent on the tumor microenvironment. In this study two 3-dimensional (3D) in vitro models were created and used to study epithelial-mesenchymal transition (EMT), a programme hypothesised to drive the metastatic process.

Materials and Methods: HCT116 colon cancer cells were encapsulated in alginate-chitosan-hyaluronan (Alg-Cs-HA) or alginate-fibronectin (Alg-Fn) matrices and co-cultured with separately encapsulated neuroblastoma (model A) or normal embryonic lung fibroblasts (model B), respectively. The internal structure of both capsule types was analysed. Cell morphology, metabolic activity, EMT-associated gene expression changes and protein expression of encapsulated cancer cells were examined.

Results and Discussion: Distinctive differences between Alg-Cs-HA and Alg-Fn capsule internal structure, different morphologies of encapsulated epithelial cells and different patterns of metabolic activity were observed. Following co-culture with fibroblasts, a more EMT-like phenotype was observed in both models (E-cadherin down-regulation and up-regulation of the mesenchymal marker, vimentin) but only in model A was the effect significant (p < 0.01, N = 2) and simultaneous up-regulation of E-cadherin transcriptional repressors, Slug and Zeb1, was observed. Analysis of matrix metalloproteinases (MMPs), involved in extracellular matrix (ECM) remodelling and cell migration, revealed significant MMP2 up-regulation (p < 0.05) and MMP9 on day 20 in both models. Upon co-culture, both models showed somewhat reduced expression of integrin α5, a receptor mediating cell-ECM interactions. Altered morphology of adherens junctions and strong MMP2 protein expression was revealed by immunostaining of cryo-sections of encapsulated cancer cells.

Conclusion: Two tumour microenvironment models used in this study, rich in different ECM components and containing two types of stromal fibroblasts, induced different changes in the epithelial phenotype of cancer cells. This 3D in vitro approach may help to understand how cancer cells interact with both adjacent and distant tissues and can potentially be used to provide in vitro models for therapeutic target validation, in particular for approaches targeting EMT or the mesenchymal phenotype, prior to in vivo studies.

Autophagic and Apoptotic Mechanisms of Death Induced by Sessbania Grandiflora Flower in Human Leukemic Cells

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Background: Ethnobotanical knowledge coupled with rationale-driven scientific research has formed an important facet of anti-cancer drug discovery because medicinal plants have a very long history of safe consumption, and bioactive compounds obtained from them are normally non-toxic or less toxic to humans. Sessbania grandiflora, a small tree, is widely used in Indian traditional medicine for the treatment of a broad spectrum of diseases. The present study provides evidence for the antiproliferative effect of S. grandiflora flowers (SG) extract in leukemic cell lines and delineates the underlying involvement of various autophotic and apoptotic pathways.

Material and Methods: Cytotoxicity of SG was checked in U937 cells by MTT assay along with flow cytometric measurement of reactive oxygen species using CMH2DCFDA, phosphatidylserine exposure by Annexin-V-FITC, mitochondrial membrane potential using JC-1 and western blot analysis of alteration in levels of autophotic and apoptotic proteins. Transmission electron microscope and fluorescence microscope were used to identify the formation of acidic vesicular organelles (AVO) during autophagy. Rate of oxygen consumption was polarographically measured with a Clark-type oxygen electrode. Caspase activity was measured spectrophotometrically. DNA degradation and nuclear morphology were determined by confocal microscopy and cell cycle analysis by flow cytometry.

Results: SG exhibited an IC50 of 18.6 g/ml in U937 cells. Inhibition of growth is associated with increased ROS generation which enhanced Annexin-V positivity through stimulation of pro-apoptotic proteins and concomitant inhibition of anti-apoptotic protein expressions producing mitochondrial disruption and the release of cytochrome-c which initiate caspase activation. Additionally, autophagic vacuoles formation clearly observed after treatment with SG and accompanied by LC3 processing and increased Atg protein expression levels. These phenomena culminated in DNA fragmentation, cell cycle arrest and further investigation cell death.

Conclusions: Taken together, the findings suggest that both apoptosis and autophagy contribute to the antileukemic activity of SG. Thus, SG merits consideration and further investigation as a therapeutic option for the treatment of leukemia.

Inhibition of Heat Shock Protein (Hsp) 27 Potentiates the Suppressive Effect of Hsp90 Inhibitors in Targeting Breast Cancer Stem-like Cells

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Background: Heat shock protein (Hsp) 90 is an ATP-dependent chaperone and its expression has been reported to be associated with poor prognosis of breast cancer. Cancer stem cells (CSCs) are particular subtypes of cells in cancer which have been demonstrated to be important to tumor initiation, drug resistance and metastasis. In breast cancer, breast CSCs (BCSCs) are identified as CD24-CD44+ cells or cells with high intracellular aldehyde dehydrogenase activity (ALDH+). Although the clinical trials of Hsp90 inhibitors in breast cancer therapy are ongoing, the BCSC targeting effect of them remains unclear.

Materials and Methods: The activity of Hsp90 was inhibited by specific inhibitors, geldanamycin (GA) or 17-desamethyl-17-N,N-dimethylaminoethyl-17-demethoxygeldanamycin (17-DMAG). The activity of Hsp27 or heat shock factor-a was inhibited by quercitin or KNK437, respectively. The changes of the population of BCSCs in human breast cancer cells were determined by fluorescence-based ALDH activity assay and flow cytometric analysis. The cell proliferation or migration was determined by methyltetrazolium assay. The drug resistance and metastasis were determined by transfecting the non-small cell lung cancer cells with the ALDH+ human breast cancer cells. GA or 17-DMAG could suppress ALDH+ breast cancer cells in a dose dependent manner. We are interested in the
Unravelling the Tumour-stroma Microenvironment of Late Stage Ovarian Cancer

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Background: Ovarian cancer is the most lethal of gynecological diseases with most women diagnosed at an advanced stage when tumour cells have spread into the peritoneum. The tumour-associated stroma is known to promote cancer progression, but the precise interactions between cancer cells and their stromal microenvironment are still poorly understood. Cancer-associated proteases like kallikrein-related (KLK) peptidases initiate peritoneal invasion via proteolytic cascades, degradation of extracellular matrix (ECM) proteins and integrin signalling implicated in cell survival and proliferation. We aimed to identify mediators of metastasis by employing a bioengineered 3-dimensional (3D) in vitro approach that mimics the tumour-stroma microenvironment of late stage ovarian cancer.

Material and Methods: We have established an integrated 3D co-culture model of epithelial (ovarian cancer) and mesothelial (stromal) cells, which we have analyzed by confocal and electron microscopy as well as proliferation assays. In patients, ovarian cancer cells present in multicellular spheroids that accumulate in the ascitic fluid and attach to thestromal peritoneal layer. To replicate this interaction, cancer spheroids were grown within polyethylene glycol-based hydrogels that comprise ECM features due to incorporation of electrospun-fabricated polycaprolactone meshes that allowed attachment of stromal cells representing the peritoneal lining. A whole human genome microarray was conducted to identify genes differentially controlled due to KLK expression and 3D co-culture. Genes were grouped by biological processes according to Gene Ontology and pathways mapped using Ingenuity.

Results: Proliferation and imaging analyses revealed enhanced spheroid growth within biomimetic hydrogels upon KLK expression compared to controls and co-cultures with stromal cells compared to mono-cultures. More genes were differentially regulated upon 3D co-culture of KLK expressing cancer cells compared to controls and stromal cells. Regulation of biosynthesis and coagulation pathways were mostly altered in cancer cells after 3D co-culture and upon KLK expression, while inflammatory, chemotactic and migratory processes were changed in stromal cells upon 3D co-culture. The prostaglandin-endoperoxide synthase 2 (PTGS2) network, including fibroblast growth factor (FGF2), fibroblast growth factor receptor 1 (FGFR1) and vascular endothelial growth factor C (VEGFC), was upregulated in cancer and stromal cells, upon KLK expression in cancer cells, and in 3D co-cultures of stromal cells compared to mono-cultures.

Conclusions: Using this integrated 3D approach, we have unravelled pathways that may be important in the aetiology of ovarian cancer metastasis and highlights the importance of tumour-stroma interactions in ovarian cancer progression.

[115] Detection of Circulating Tumor Cells on the Basis of Cytomorphology, Immunofluorescence and in Situ Hybridization with the Aid of a “Robotized Microscope”: from Bench to Bedside

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Background: Despite major advances in research and therapy, cancer continues to be the second-leading cause of death among the industrialized countries and metastases are the most important factor causing the adverse prognosis of carcinoma patients. Cancer cells can detach and disseminate in the blood stream from the primary tumor very early in the tumor progression and the detection of rare circulating tumor cells (CTCs) in cancer patients is of incredible utility to monitor the progression and assess response to treatment. CTCs have been detected in a majority of epithelial cancers, including those from breast, prostate, lung and colon both before the primary tumor is detected and even when the carcinoma recurs. Currently, the methods for detecting CTCs include cytometric and nucleic acid based approaches. In our laboratory we use a filtration-based technique to isolate CTCs preserving the cellular shape and morphology and we develop an assay that combines enumeration and genotyping of CTCs, based on a combination of antibody detection, FISH and automated fluorescence microscopy.

Material and Methods: The assay comprises cell enrichment, by filtration through an 8 μm pores membrane, immunofluorescent staining for the detection of the epithelial marker EpCAM (AUA1), cytokeratins 7 and 8 (CAM 5.2) and CD45 for the negative selection of leukocytes. We also use a combination of FISH probes for the detection of cancer-related genes. The images acquisition is performed with the ikonoskop robotic microscope, a fully automated image acquisition and display system specific for rare cell identification.

Results: We spiked into the blood of a healthy donor a known number of cells of the prostate carcinoma cell-line LNCaP and processed the samples through the filtration and detection protocol. We were able to collect/detect the CTCs with high sensitivity and specificity because of the flexibility of the method: we identify the CTCs both on physical cell morphology and immunological properties and with a combination of antibody and/or FISH probes.

Conclusion: Maintaining the integrity of the tumor cells detected, our method allows further analysis of cell morphology and genotype. The combination of antibodies and FISH probes, and the possibility of the morphological evaluation, holds great promises for screening, early detection of recurrence/relapse and evaluation of treatment response.

[117] TAp73-mediated Transcriptional Activity and Apoptosis are Influenced by Diverse P53 Isoforms

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Introduction: The p53 activities are due, at least in part, to its ability to form oligomers that bind to specific DNA sequences and activate transcription. Since some mutant p53 proteins, as well as ΔNp73 isoforms form a homo-oligomeric complex with TAp73, we asked whether TAp73 isoforms can do the same and potentially act as dominant-negative inhibitors of TAp73. Moreover, it was already found that some isoforms form complex with ΔNp53 and some of them inhibit p53 tumor suppressor functions.
Material and Methods: The p53 null human cell line H1299 cells were used as a model. Proteins were extracted and western blot was performed by standard methods. The complex formation of p53 isoforms was determined by communoprecipitation assay. Transcriptional and apoptotic function of p53 were measured by Dual-Glo Luciferase Assay or Annexin-V-FLUOS staining kit, respectively. The half lives of different p53 isoforms have been determined using pulse chase method with cycloheximide.

Results and Discussion: Coimmunoprecipitation assay has shown that all six p53 isoforms examined can form complexes with p53: isoform, while only D133p53 isoforms (η, ι, and γ) can interact with p53. All p53 isoforms counteract the p53 transactivation function but with different efficiency and in a promoter-dependent manner. Furthermore, apoptotic function of p53 was augmented by coexpression of p53b, while ι133p53a and ι133p53b inhibit its apoptotic activity most efficiently. The half lives of different p53 isoforms have shown that p53i is shorter than ι133p53a; the longest half life. Inhibitory interactions of two proteins in complex often lead to their stabilization. However, only three isoforms (ι133p53a, ι133p53b, and ι40p53b) stabilize the TAp73.

Conclusion: p53 isoforms may modulate p53 family tumor suppressor activities, and the expression ratio between members of the family is probably the most important factor in determining the cell fate and outcome.

[118] Pro-Inflammatory Cytokine-Induced Homeobox Gene, Isx, Regulates Tumorigenic Activity and Survival in Hepatocellular Carcinoma L.T. Wang, S.H. Hsu 1. Kaohsiung Medical University, Graduate Institute of Medicine, Kaohsiung, Taiwan

Background: Hepatocellular carcinoma (HCC), a chronic inflammation associated malignancies, leads third cancer deaths each year worldwide; so far, the regulatory mechanism is still unclear.

Material and Methods: A total of pair samples (tumor mass and adjacent normal tissues) from 119 HCC patients and 11 non-HCC patients were collected to be enrolled in this cohort study and sera were analyzed as routine laboratory tests. Through real-time quantitative PCR, immunostaining and promoter binding assay in vitro and in vivo, the correlation of IL-6, Isx and cyclin D1 were addressed.

Results: Isx, an IL-6-induced homeobox gene, showed a tumor-specific expression pattern in pathological analysis and high correlation to HCC patients’ survival time, tumor size, numbers and stage progression. Enhanced expression of Isx accelerated cell proliferation and tumorigenic activity in hepatoma cells via cyclin D1 expression in vitro and in vivo. The abating of Isx expression accelerated cell proliferation and transdifferentiation activity in vitro and in vivo. Also, up-regulated Isx expression also showed a high correlation to the elevation of cyclin D1 in human hepatoma tumors infected with HBV/HCV.

Conclusion: These results indicated that Isx, as a potential processive marker, plays an important regulatory role in hepatoma progression and is a worthwhile therapeutic target for future investigation.

[121] Functional Interaction of Ugene and EBV Infection Mediates Tumorigenic Effects L.T. Wang, S.H. Hsu 1. Kaohsiung Medical University, Graduate Institute of Medicine, Kaohsiung, Taiwan

Background: Epstein–Barr virus (EBV) infection is associated with many human neoplasms, in which EBV-associated latent membrane protein-1 (LMP1) appears to be critical, but its exact oncogenic mechanism remains to be defined. To this end, our initial microarray analyses identified a LMP1-inducible gene, Ugene, characterized as a binding partner for uracil DNA glycosylase 2, which is highly expressed in malignant colon cancer. In this report, we found that Ugene, designated herein as LMP1-induced protein (LMPIP), was induced, in a time-dependent manner, in EBV-infected peripheral blood mononuclear cells and LMP1-transfected 293 cells.

Material and Methods: Functionally, when compared with mock-transfected cells, overexpression of LMPIP in nasopharyngeal carcinoma (NPC) cell lines resulted in a decrease in reactive oxygen species production and maintained mitochondria membrane potential ($\Delta$Ψm) loss induced by H$_2$O$_2$.

Results: The NPC cells transfected with LMPIP also showed a decrease in G1 population and an increase in the cell population in S phase, while the number of cells in multiphase phase increased. Significantly, NPC cells with LMPIP knock-down also showed a decrease in tumorigenic and transforming activity induced by ectopic LMP1 expression determined by analyses of soft agar foc and tumor size in nude mice. Further, elevated LMP1 expression was also noted in cytoplasm and nuclei in EBV-infected NPC tumor cell mass and non-EBV-infected tumor cell lines.

Conclusions: These results suggested that LMPIP may have an important mediator role in EBV-mediated neoplasia and may serve as a new target for therapy of tumors induced by EBV infection.

[122] Poly(I:C) Stimulation Influences the Expression of Calretulinic, Profilin-1 and Heterogeneous Nuclear Ribonucleoprotein A1 in a Human HNSCC Cell Line: A Proteomic Study T. Matjevic Glavan1, J. Pavlic1, Rudjer Boskovic Institute, Department of Molecular Medicine, Zagreb, Croatia

Introduction: Poly(I:C) has been formerly known to be an interferon inducer but the mechanism of its action was not revealed until the discovery of Toll-like receptors (TLRs). TLRs are transmembrane proteins that recognize conserved molecular motifs of viral and bacterial origin and initiate innate immune response. Recent studies have shown that they are also expressed on tumor cells but their role in these cells is still not clear. TLR3 recognizes double stranded RNA [poly(I:C)] and is primarily involved in the defense against viruses similarly as other two cytoplasmic receptors, melanoma differentiation associated gene 5 (MDA5) and retinoic acid inducible gene 1 (RIG-I), that also recognize dsRNA. Poly(I:C) binding initiates the activation of transcription factors NF-κB, IRF family members and AP-1, which can induce wide cascading effect on the cell and consequently activate many cellular processes.

Material and Methods: In order to widen the current knowledge of TLR3 target genes, we have treated HNSCC cell line S28 with poly(I:C) and used the proteomic approach. We have confirmed the obtained results by western blot.

Results and Discussion: In this study we have discovered 15 differentially expressed proteins, mostly connected with protein metabolic processes. Furthermore, we have confirmed that calretulinic, profilin-1 and heterogeneous nuclear ribonucleoprotein A1 (HNRNP1), proteins which have been shown previously to be involved in processes connected with tumor progression, are differentially expressed after poly(I:C) stimulation. In addition, we have shown that HNRNP1 is noticeably over-expressed after the inhibition of MDA5 and RIG-I expression.

Conclusion: Since poly(I:C) has been studied and utilized in tumor therapy as an immunostimulatory adjuvant, our results indicate that further studies are needed before the clinical application in order to prevent possible side effects.

[123] Integrin-mediated Adhesion and Signaling are Decoupled and Controlled by Their Transmembrane Domains U. Reuning1, M. Mueller1, J. Opfer1, L. Volkhardt1, L. Brunie1, D. Boettiger2, H. Kessler1, K.E. Gottschalk2. 1Klinikum Rechts der Isar, Obstetrics & Gynecology, München, Germany, 2University of Pennsylvania, Experimental Physics, Ulm, Germany, 3University of Pennsylvania, Institute for Medicine & Engineering, Philadelphia, USA, 4Technische Universität Muenchen, Chemistry Department, München, Germany

Background: Cell adhesion and migration are biological events involving integrins as main adhesion and signaling receptors. Integrins are heterodimers non-covalently composed of an α- and a β-subunit. Each of which encompasses a large extracellular domain, a single-pass transmembrane domain (TMD), and a short cytoplasmic tail. After binding to extracellular matrix ligands, integrins trigger bi-directional cell signaling affecting cell migration, apoptosis, and cell differentiation. Therefore, integrins adopt multiple conformational and functional states. Here, we explore the role of integrin conformation for integrin activation.

Materials and Methods: Integrin $\alpha v \beta 3$ chimera encompassing the strongly dimerizing TMD of glycoprophorin A (TMD-GpA) were generated by in vitro site-directed mutagenesis. In addition, we point-mutated the central GxxG dimerization motif of GpA-TMD to GxxG (TMD-GpA-a), known to induce TMD uncoupling. We analyzed the effects of these integrin $\alpha v \beta 3$ chimeric constructs on cell adhesion by atomic force microscopy and spinning disc tests. Ligand binding tests were conducted by FACS analysis. Cell migration was studied by wound healing assays. Integrin $\alpha v \beta 3$-related signaling was monitored by the activation state of signaling molecules, such as the focal adhesion kinase (FAK), mitogen activated protein kinases (MAPK), and protein kinase B (Akt).

Results: Despite tight TMD dimerization, TMD-GpA provoked strong cell adhesion and did not promote cell migration and signaling. In contrast, TMD-GpA-a with uncoupled TMD provoked high adhesion strength and also triggered cell signaling and migration. These findings correspond well to a three-state model of integrin activation: a resting state is activated by intracellular ligands to an intermediate state without integrin TMD separation. Subsequent binding of ligands then triggers TMD uncoupling which induces intracellular signaling and integrin linkage to a new conformation as prerequisite for cell migration. This state is mimicked by constitutively active TMD-GpA-a.

Conclusions: Integrin-mediated cell adhesion may be decoupled from cell signaling and migration based on TMD conformational states in an allosteric physics, biology, and bioinformatics.
Role of Mesenchymal Stem Cells as a Co-conspirator for Tumor Progression in Syngeneic Rat Osteosarcoma Model: A Comparative Analysis of Gene Expression Profiles for Tumor Progression

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Introduction: Recent reports indicated that the mesenchymal stem cells (MSCs) might play an important role in tumor development, however the role of MSCs still remains uncovered in most sarcomas. We investigated the role of MSCs in sarcoma progression with comparative analysis of gene expression profiles based upon the results of in vivo experiments in rat osteosarcoma model.

Materials and Methods: Two components of experiments have been performed using the cell lines of rat osteosarcoma COS1N1 established from the chemically induced osteosarcoma in F344 rats and rat MSCs isolated from syngeneic rat femur bone marrow. In vivo studies were performed as the simultaneous co-implantation of both cells into subcutaneous tissues and subcutaneous inoculation of osteosarcoma cells followed by subsequent intravenous injection of MSCs intermittently. The gene expression profiles using Agilent gene expression array were compared between MSCs and osteosarcoma cells to identify the factors possibly involved in the process of tumor progression.

Results and Discussion: Simultaneous co-implantation of both cells showed higher incidence of tumor formation and tumor growth rate in early phase compared to osteosarcoma cell inoculation alone. Intravenous MSCs injection after subcutaneous inoculation of osteosarcoma cells enhanced the formation of lung metastatic nodules in the group with MSCs injection compared to the group without MSCs, while no difference was observed in subcutaneous tumor growth between those groups. The pathway analysis from comparative gene expression profiles identified the pathways involved in cell cycle acceleration and DNA repairs were up-regulated in osteosarcoma cells, while genes involved in focal adhesion, cytokine–cytokine receptor, chemokine signaling and extracellular matrix–receptor pathways such as CAMs-integrins, CCs-CCRs and MMPs, mostly related to tumor progression such as invasion and metastasis, were up-regulated in MSCs (Table I), suggesting possible participation of MSCs in the tumor progression through the cell–cell and cell–matrix interactions as a co-conspirator.

<table>
<thead>
<tr>
<th>Pathways with higher incidence of strongly expressed genes in:</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat MSCs cell adhesion</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ECM–receptor interaction</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cytokine–cytokine receptor interaction</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Chemokine signaling</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rat Sarcoma DNA replication</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>0.0005</td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>0.0020</td>
</tr>
</tbody>
</table>

Conclusions: We hypothesize that MSCs could be a potential co-conspirator for osteosarcoma cells to enhance the ability of settlement and colonization leading to early onset of growth and metastasis. The activated pathways in MSCs may be involved in tumor progression through the cell–cell and cell–matrix interaction, potentially compensating the tumor cell ability and cultivating the microenvironments for tumor cells.

Fundamental Cell Regulation by ADAR1 Enzyme is Lost in Metastasis by MicroRNAs to Promote Malignancy

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Introduction: We present a substantial new evidence for ADAR1 fundamental role as regulator of cancer cell phenotype and unravel the underlying genomic and epigenetic events that are responsible for loss of ADAR1 expression in cancer cells, which subsequently facilitate the acquisition of an aggressive phenotype.

Material and Methods: A wide variety of in vitro and in vivo assays and molecular techniques (FACS, Real time quantitative PCR, Western blot, microarray analysis, Luciferase reporter assay, quantification of methylation levels, TMA and human xenografts model) has been performed based on 32 metastatic primary cultures, 4 metastatic breast cancer model cell lines were then ADAR1 expression was evaluated and accordingly ADAR1-manipulated cell system was constructed to enable further investigation of ADAR1 function and regulation during cancer progression.

Results and Discussion: In-depth studies on melanoma samples and progression tissue microarrays point on a substantial ADAR1 downregulation during metastatic transition. Accordingly, we found that ADAR1 suppresses several cancer features, as its downregulation alters cell morphology, facilitates cell-cycle and proliferation in vitro, and dramatically enhances the tumorigenicity in vivo. We show that ADAR1 controls the expression of >100 microRNAs, which regulate hundreds of genes that account for the observed phenotype. A series of truncation mutants establishes novel RNA-editing-independent roles for the short and long ADAR1 isoforms in controlling the nuclear and cytoplasmic processing steps of miRNA biogenesis, respectively, independently of Dicer or Drosha. Cancer cells silence ADAR1 by overexpressing miR-17 and miR-432, both of which directly target ADAR1 and operate additively. Both of their genomic sequences are frequently amplified to increase expression, but uniquely, an aberrant hypomethylation of the imprinted DLK1-DIO3 region in chromosome 14 accounts for the miR-432 overexpression.

Conclusion: The novel ADAR1-dependent and RNA-editing-independent roles as well as the miRNA-mediated regulation mechanisms of ADAR1 expression, strongly points on a central involvement of ADAR1 in cancer progression and provide new insights on the process of cancer development with potential implications for future translational medicine.

Integrating Transcriptomic and Genetic Approaches to Identify Novel Mediators of Breast Cancer Metastasis

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Background: It is widely accepted that cancer metastasis is the main cause for the majority of breast cancer patient deaths. Despite recent advances in technology and the identification of various metastasis gene signatures, the molecular mechanisms that control dissemination of cancer cells to secondary tissues remain largely elusive. In this study, we take advantage of a human breast cancer cell line model system which consists of four isogenic cell lines that exhibit various properties ranging from non-tumorigenic to metastatic.

Materials and Methods: The strategy is based on gene expression profiling of the model breast cancer cell lines using DNA microarrays. Expression analysis was followed by in vitro validation and application of meta-analysis with relevant microarray data from human breast tumours to identify potential metastasis promoting or suppressor genes. The breast cancer model cell lines were then stably transduced with the luciferase gene and the selected candidate genes were genetically manipulated in these cells. In vivo metastasis assays coupled with whole-body bioluminescence imaging were used to assess the functional role of particular genes.

Results: Using microarray gene expression analysis we initially identified four major gene clusters which consist of 55 candidate metastasis promoting and 124 candidate metastasis suppressor genes. Meta-analysis from publically available microarray data sets indicated that the expression pattern for 14 candidate metastasis suppressors and 5 metastasis promoting genes significantly correlates with the expression from primary compared to metastatic breast tumours and from patients with good versus poor outcome. Eight selected genes were further genetically manipulated in the luciferase expressing metastatic cell line, by either retroviral-mediated gene transduction or lentiviral-mediated shRNA knock-down. In vivo metastasis assays were conducted in NOD/SCID mice and metastatic dissemination of these cell lines was assessed using the IVIS bioluminescence imaging system. We found that a subset of the selected genes significantly affected the ability of breast cancer cells to metastasize to the lungs.
Conclusion: The combination of genome-wide gene expression profiling in defined isogenic experimental cancer models with in silico meta-analyses and mathematical modeling of identified genes can be a powerful tool for the identification of novel therapeutic targets against metastatic breast cancer.

The Effect of a Novel Daunomycin-conjugate With an ErbB2 Receptor-binding Peptide on the Protein Expression Profile of HL-60 Cells

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Background: Daunomycin (Dau) is one of the most efficient chemotherapy agents used in leukemia treatment. Due to the lack of selectivity it has severe side effects such as cardiotoxicity. We have demonstrated earlier that its conjugation to peptide/protein carriers (e.g.: oligoarginine, branched chain polypeptides) modifies its effect. ErbB2 receptor is overexpressed on several tumor cells, therefore it is a potential target in cancer chemotherapy. A tumor-specific peptide (LTVSPWY) derived from a phage peptide library was described as an ErbB2 receptor-binding peptide. This peptide has been used in our laboratory to prepare a novel conjugate with Dau (Orbán et al., Bioconj. Chem., 2011).

Materials and Methods: Dau=Oaa=LTVSPWYNH4 conjugate and control compounds were prepared and chemically characterized. In vitro cytostatic effect of the compounds was determined with MTT-assay and cellular uptake properties were studied by flow cytometry. The mechanism of cellular uptake was studied using various inhibitors. For these experiments tumor cell lines with different expression levels of ErbB2 receptor were applied. Protein expression profile of cells treated with Dau or its conjugate was determined and compared with that of non-treated cells using two-dimensional gel electrophoresis. Proteins with significantly different expression were identified with nano-LC-MS/MS.

Results: We observed significant in vitro cytostatic effect of the new conjugates on the studied cell lines. The highest cellular uptake ratio was observed on HL-60 human leukemia cells. The results of the proteomic studies with these cells clearly indicate the altered expression of a limited number of proteins. The expression of cytoskeletal, metabolic proteins and proteins involved in signaling processes were altered due to the treatment of the cells with free drug or the daunomycin-conjugate. Details will be discussed in relation with the potential mechanism of action.

Conclusion: A new daunomycin-conjugate containing Dau and peptide LTVSPWY showed in vitro cytostatic effect and cellular uptake ratio. Protein expression profile of HL-60 human leukemia cells was different after treatment with Dau or the conjugate. The analysis of proteomical data in combination with inhibition experiments leads to the understanding of the mechanism of action.

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Pancreatic Cancer Microenvironment: Role of Macrophages

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Background: Pancreatic cancer remains the most aggressive malignancy of all human cancers. Patients have an extremely poor prognosis, with 5-year survival less than 5% and a median survival of 6 months. Pancreatic cancer is characterized by infiltrating tubular units embedded in desmoplastic stroma which is rich in immune cells, endothelial cells and fibroblasts. This heterogeneous microenvironment creates great conditions for tumor development. The question arises whether infiltrating macrophages may be responsible for cancer cells detachment and metastasis formation.

Aim: To evaluate enzymatic activity and influence of macrophages in pancreatic cancer microenvironment.

Materials: Tumor tissue samples were obtained from 36 patients who underwent macroscopically curative resection. Patients had not received any preoperative radio- or chemotherapy. Tissue specimens were analyzed with immunohistochemistry and expression of macrophages using antibodies against CD3, CD63, elastase, HGF, c-Met, EGF and EGFRI. Gelatin zymography and zymography in situ was used for evaluation of MMP2 and MMP9 activity.

Results: Immunostaining showed CD68 positive macrophages infiltrates around cancer nests and also in surrounding tissue. Their number was significantly higher in group with lymph node metastases. Zymography revealed that both active metalloproteinases were present in pancreatic cancer tumor tissue.

Conclusion: The obtained results indicate that immune cell enzymatic activity have impact on tumor progression. Abundant growth factors expression and MMPs activity might support EMT process which seems to be pivotal in pancreatic cancer.

Results of the Primary Treatment of Patients With Serous Ovarian Cancer Stage 2 in Relation to Degree of Process Differentiation

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Background: The ovarian cancer is on the third place in the structure of oncological diseases of the reproductive system in women. Nowadays the question about significance of traditional clinical-morphological factors of prognosis in patients with ovarian cancer remains to be problematical. It is evident that the stage of disease is the leading factor on the basis of which the plane is developing both for diagnostic and therapeutic measures.

Purpose: To evaluate results of the primary treatment of the patients with serous ovarian cancer stage 2.

Material and Methods: In our investigation the ovarian cancer stage 2 was diagnosed in 58 women with ovarian cancer, of them 11a = 9, 1lb = 35, 1lc = 14 patients. The 5-year-survival was 52.8±8.9%, without signs of progression of disease = 50.9±9.8% patients. The average age of patients in this group was 56.3±3.1 years. The age content of the patients from group II was following: under 45 years = 5(8.6%) patients; at the age of 45−59 years = 27(46.6%) and 60 years and from 60 years to 75 − 25 (44.8%) patients. During assessment of the general morbidity we found that top the 5 years of follow up were alive 63.1±11.6% patients of young age, 50.7±14.5% patients were of middle age and 46.9±15.1% − aged patients (p < 0.05). Thus, we were unable to reveal correlation between long-term results of treatment of patients with ovarian cancer stage II and age factor.

Results and Discussion: According to the degree of tumor differentiation the patients with ovarian cancer stage II were divided by the following way: low degree of tumor differentiation in 29 (50%) patients, moderate differentiated tumors in 25 (43.1%) and highly differentiated ovarian cancer in 4 (6.9%) patients. These results confirmed that low degree of tumor differentiation is unfavorable prognostic factor in the patients with ovarian cancer. Histological investigation of the removed material showed that in 39 (67.2%) patients tumor had structure of serous adenocarcinoma, in 8 (13.0%) − mucinous adenocarcinoma, in 13 (22.4%) 0 endometrioid adenocarcinoma. All 6 patients with mucinous adenocarcinoma were alive after 5 year-observation without signs of disease progressing. In comparison of the long-term results of treatment of patients with serous adenocarcinoma and patients with endometrioid tumors the parameters of 5-year survival were 48.9±7.3% and 68.2±7.4%, respectively (p < 0.05). There were reliable differentiation of free survival: during 5 years without signs of basic disease progress in 42.3±8.3% of patients with serous neoplasms and 67.3±8.9% patients with endometrioid adenocarcinoma. (p <0.05).

Conclusion: Thus, analysis of the long-term results of treatment of patients with ovarian cancer stage II showed that serous morphological variant of tumor is prognostically unfavorable.
Metabolic Responses to VEGF Inhibition in Patient-derived Breast Cancer Xenografts

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Introduction: Bevacizumab targets angiogenesis through VEGF inhibition, thereby causing changes in existing tumor vasculature and architecture, and inhibiting tumor growth. Since acquired resistance to bevacizumab treatment occurs frequently, there is a need for noninvasive therapy monitoring methods. Most studies have focused on the vascular effects, but using magnetic resonance spectroscopy (MRS) to study metabolic effects may also provide information on the response to treatment. The objective of this study was to assess changes in the metabolic profiles in two patient-derived breast cancer xenografts after treatment with bevacizumab, using ex vivo MRS.

Material and Method: Mice with basal-like (n = 5) and luminal-like (n = 5) xenografts were treated with bevacizumab (5 mg/kg ip). Tumor tissue was frozen after 3 or 7 days of treatment and flash frozen in liquid nitrogen. The metabolic profile was determined by high resolution magic angle spinning (HR MAS) MRS and compared to the profile of untreated control tumors. The MR spectra were analyzed using principal component analysis (PCA) to detect patterns within the metabolic response to treatment. Therapeutic efficacy was evaluated by histopathologic evaluation of microvessel density (MVD) and proliferating MVD (pMVD).

Results and Discussion: In basal-like xenografts, bevacizumab caused a significant reduction in both MVD and pMVD, indicating response to bevacizumab treatment. No significant change was seen in luminal-like xenografts. Using PCA, spectra from basal-like xenografts treated with bevacizumab were clearly separated from control samples. The two principal components accounted for 90% and 6% of the difference between treated and control tumors. The first principal component was associated with an increase in mobile MR-visible lipids, a marker for apoptosis. The second principal component was related to a decrease in GPC:PCho ratio, showing that basal-like xenografts treated with bevacizumab had lower GPC and higher PCho concentrations than controls. For the luminal-like xenografts, no clear separation was found, reflecting the absence of response in the vascular parameters.

Conclusion: Changes in metabolic profile may identify early response to bevacizumab treatment in breast cancer, due to increased mobile lipid content or shifts in GPC:PCho ratio. This could potentially be used for in vivo MRS monitoring of treatment response.

A New Type of Oncogene, SWAP-70

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Background: SWAP-70 is a phosphatidylinositol-triphosphate (PIP3) binding protein containing a PH domain at the central domain, an F-actin binding domain at the very carboxyl terminal domain, a coiled-coil domain at the carboxyl half, and a Rac1 domain at the amino terminal domain and has been shown to be involved in membrane ruffling induced by EGF.

Material and Methods: Mutations were introduced into the SWAP-70 gene and the mutant genes were introduced normal cells and their phenotypes were observed.

Results: We noticed that mouse embryo fibroblasts (MEFs) lacking SWAP-70 grow more slowly than the wild type MEFs, suggesting that SWAP-70 is regulating cell growth. We found that MEFs lacking SWAP-70 transformed by V-Src oncogene are far less aggressive than the ones bearing SWAP-70. And finally, we found that one of the SWAP-70 mutants, SWAP-70-374, can transform MEFs in vitro and is resistant to the treatment, a coiled-coil domain at the amino terminal domain and has been shown to be involved in membrane ruffling induced by EGF.

Conclusion: In pancreatic cancer cells, melanoma cells and genetically defined mouse embryonic fibroblasts (MEFs), we used the proapoptotic inhibitor bortezomib for stabilisation of the MYC protein. A further transcriptional analysis revealed that the introduction of the pro-apoptotic BCL2 family members could be reverted after MYC inhibition using RNAi and small molecule inhibitors.

Bortezomib induced apoptosis was blocked with MYC specific siRNAs and the MYC/MAX heterodimerisation interfering inhibitor 10058-F4. Consistent, deletion of both MYC alleles by tamoxifen treatment of 3T9-flCreER MEFs leads to inhibition of bortezomib-induced apoptosis. We further analyzed the expression of the pro-apoptotic BCL2 family members and observed mRNA and protein induction of the BH3-only protein BIM in NOXA deficient MEFs. BIM was also induced in tumor cells by bortezomib treatment in a MYC-dependent fashion. Furthermore, the contribution of BIM towards bortezomib-induced apoptosis was demonstrated with RNAi. At the proximal NOXA as well as at the proximal BIM promoter we could detect euchromatin marks, like tri-methylated lysine 4 of histone H3 (H3K4me3). In response to proteasome inhibition, we found a direct MYC binding to both promoters, with subsequent preferentially acetylation of histone H4, recruitment of RNA polymerase II and transcriptional activation of both genes.

Conclusion: Taken together, NOXA and BIM are direct MYC target genes, acting together to execute MYC-dependent apoptosis.

Implication of Brush Border Membrane Lipid Composition and Fluidity in Altered Alkaline Phosphatase Activity in Renal Cell Carcinoma

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Background: The present study was conducted to find out the role of membrane lipid fluidity and membrane composition in altered activity of alkaline phosphatase (ALP) in brush border membrane (BBM) from renal cell carcinoma (RCC).

Materials and Methods: Histopathologically confirmed 30 cases of RCC were included in the present investigation.

Results: A significant decrease was observed in the activity of alkaline phosphatase in RCC isolated from RCC as compared to BBM isolated from normal renal parenchyma. Antecedent studies have suggested that ALP is directly correlated with membrane lipid composition and fluidity. In view of this, we determined the role of membrane lipid composition and fluidity of BBM in decreased activity of ALP in BBM from RCC. The fluorescent anisotropy (dDPH) and polarization (pDPPH) of diphenyl hexatriene were significantly decreased. These parameters are inversely proportional to membrane fluidity. Therefore, this finding was suggestive of increased membrane fluidity in BBM isolated from RCC.

Conclusions: SWAP-70 is a novel type of oncogene, which regulates a novel signaling system that is important for cell growth.

MYC Transcription Control of NOXA and BIM to Trigger Apoptosis

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Background: The MYC oncoprotein directs a variety of tumor promoting programs in tumor cells. Besides proliferation MYC also triggers apoptosis under distinct conditions. The molecular mechanisms of MYC induced apoptosis are still incomplete understood. Master regulators of cell intrinsic apoptosis are the members of the BCL2 protein family. So called BH3-only proteins sensitize for apoptosis induction.

Materials and Methods: In this study we analysed MYC-dependent apoptosis in pancreatic cancer cells, melanoma cells and genetically defined mouse embryonic fibroblasts (MEFs). We used the proteasome inhibitor bortezomib for stabilisation of the MYC protein. A further transcriptional analysis revealed that the introduction of the pro-apoptotic BCL2 family members could be reverted after MYC inhibition using RNAi and small molecule inhibitors.

Results: Bortezomib induced apoptosis was blocked with MYC specific siRNAs and the MYC/MAX heterodimerisation interfering inhibitor 10058-F4. Consistent, deletion of both MYC alleles by tamoxifen treatment of 3T9-Myc0/1 MEFs leads to inhibition of bortezomib-induced apoptosis. We further analyzed the expression of the pro-apoptotic BCL2 family members and observed mRNA and protein induction of the BH3-only pro death player NOXA after 4 hours of bortezomib treatment. The induction of NOXA could be reverted after MYC inhibition using RNAi and small molecule inhibitors, suggesting a direct transcriptional role for MYC. Consistent with a function of NOXA in the bortezomib response, NOXA deficient MEFs were partially protected from bortezomib-induced cell death. Interestingly, bortezomib induced apoptosis of NOXA deficient MEFs was completely blocked by proteasome inhibition. We conclude that cooperation of the detection and execution function of the proteasome BLM in NOXA deficient MEFs. BIM was also induced in tumor cells by bortezomib treatment in a MYC-dependent fashion. Furthermore, the contribution of BIM towards bortezomib-induced apoptosis was demonstrated with RNAi. At the proximal NOXA as well as at the proximal BIM promoter we could detect euchromatin marks, like tri-methylated lysine 4 of histone H3 (H3K4me3). In response to proteasome inhibition, we found a direct MYC binding to both promoters, with subsequent preferentially acetylation of histone H4, recruitment of RNA polymerase II and transcriptional activation of both genes.
Mitochondria-mediated Reactive Oxygen Species May Relate to Docosahexaenoic Acid-induced Apoptosis in Human Cervical Cancer Cells

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Introduction: Reactive oxygen species (ROS) produced by docosahexaenoic acid (DHA) have an important function in cancer cell death. However, the exact mechanism of ROS production, after DHA stimulation, is not clearly understood. Here, we determined that elevated levels of ROS generated by mitochondrial respiration is directly associated with DHA-induced cervical cancer cell death.

Material and Method: The effects of DHA on cell proliferation and cell cycle were examined by MTT assay and FACS. DHA-induced apoptosis was analyzed using TUNEL assay, caspase activity assay, and western blot. Dihydroethidium (DHE) was used for reactive oxygen species (ROS) measurement in cytosol. MitoSox was performed for ROS measurement in mitochondria.

Results and Discussion: The levels of caspase 3 activity, TUNEL-positive staining cells and Sub-G1 portion were markedly increased in DHA-treated cancer cells, suggesting that apoptosis is responsible for the DHA-induced cervical cancer cell death. Furthermore, DHA was able to induce both mitochondrial complex I substrate- and complex II substrate-supported mitochondrial ROS production in isolated mitochondria from rodent liver. Meanwhile, a reduction in oxygen consumption rate and an increase in mitochondrial ROS production as measured by MitoSOX were also observed in DHA-treated cancer cells, indicating that DHA can directly act on mitochondrial respiration and enhance ROS generation. The role of DHA-induced mitochondrial ROS production in apoptosis was further identified by the findings that DHA reduced the mitochondrial membrane potential, resulting in cardiopin oxidation and cytochrome c release from mitochondria, and that N-acetylcyesteine, an antioxidant almost completely blocked these processes as well as ROS production occurred in mitochondria and remarkably reversed the apoptotic cell death triggered by DHA.

Conclusion: From the results presented here, we conclude that mitochondria actively participate in the DHA-induced apoptotic cell death by the generation of mitochondrial ROS. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (2011–0006232 and 2011–0003060).

Docosahexaenoic Acid Induces Apoptosis Through Reactive Oxygen Species-dependent ERK and JNK Activation in Human Ovarian Cancer Cells

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Introduction: Although abundant experimental evidences show that the Omega-3 polyunsaturated fatty acids (ω3-PUFAs) prevent carcinogenesis, the exact molecular mechanisms of the anti-cancer actions of ω3-PUFAs in ovarian cancer remain incompletely understood. In the present study, the effectiveness of docosahexaenoic acid (DHA), a ω3-PUFA, against ovarian cancer cells was investigated.

Material and Method: Cell viability was analyzed using the MTT assay. Signaling proteins were detected by Western blot assay. TUNEL assay and FACS analysis were used for measuring apoptotic cell death. Dihydroethidium (DHE) was used for reactive oxygen species (ROS) measurement in cytosol. MitoSox was performed for ROS measurement in mitochondria.

Results and Discussion: We found that DHA induced cell cytotoxicity in three ovarian cancer cells including PA-1, MDAH2774 and ID8. DHA treatment induced the cell proliferation of PA-1 cells in a dose- and time-dependent manner. Meanwhile, DHA-treated ovarian cancer cells showed increased levels of caspase-3 activity, Annexin-V staining positive cells, TUNEL-positive cells and the portion of Sub-G1 cells, suggesting that DHA-induced cell death is mainly associated with apoptosis. Western blot and immunocytochemistry assays revealed that DHA also remarkably increased the levels of phospho-ERK and phospho-JNK in both cytosol and nucleus. Moreover, knockdown ERK and JNK by small interfering RNAs partially attenuated the apoptosis induced by DHA, indicating that ERK and JNK activation is responsible for the apoptosis in DHA-treated ovarian cancer cells. In addition, we determined that the activation of ERK and JNK was associated with the reactive oxygen species (ROS) production induced by DHA, ROS scavenger, N-acetyl-L-cysteine (NAC), almost completely blocked the ERK and JNK phosphorylation as well as the apoptosis triggered by DHA.

Conclusion: Together, these results indicate that DHA induces ROS and the ROS-dependent ERK and JNK activation is important to DHA-induced cell cytotoxicity in human ovarian cancer cells.

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011–0006232 and 2011–0003060).

Inhibition of mTor Through AMPK Activation and PI3K/Akt Inhibition is Important to Docosahexaenoic Acid-induced Cell Death in Human Non-small Cell Lung Cancer Cells

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Introduction: Over 80% of the lung cancer patients have non-small cell lung cancer (NSCLC). Although the anticancer mechanisms of omega-3-polyunsaturated fatty acids (ω3-PUFAs) have been reported in several cancers, it is still unclear in lung cancer. In this study, we have identified a novel anticancer effect of DHA, a ω3-PUFAs, on NSCLC.

Material and Method: Cell viability was analyzed using the MTT assay. Signaling proteins were detected by Western blot assay. TUNEL assay and FACS analysis were used for measuring apoptosis. Dihydroethidium (DHE) was used for reactive oxygen species (ROS) measurement in cytosol. MitoSox was used to transfect Akt gene to cells. Autophagy was detected after docosahexaenoic acid (DHA), an omega 3-PUFA, exposure as indicated by induction of LC3 expression, and formation of autophagic vacuolization.

Results and Discussion: DHA induced cytotoxicity and morphology change in A549 and H1299 NSCLC cells and was confirmed that apoptosis and autophagy are responsible for the cytotoxicity induced by DHA. The DHA-induced cell death is accompanied by an increase in AMPK activity and decrease in PI3K/Akt signaling as well as mTOR signaling molecules. Knockdown AMPK using small interfering RNAs specific for AMPK and overexpression Akt significantly enhanced the mTOR activity and attenuated the cell death caused by DHA treatment, indicating that DHA induces NSCLC cell death via the AMPK- and Akt-regulated mTOR inactivation. We also confirmed this effect of ω3-PUFAs in vivo using Fat-1 transgenic mice that are capable of producing ω3-PUFAs. When mouse lung cancer LLC cells were subcutaneously implanted into Fat-1 mice, the growth of tumor was markedly inhibited with decreases of the level of p-Akt as well as increases in TUNEL-positive staining cells and autophagic markers, compared to wild-type mice.

Conclusion: Taken together, these data suggest that the apoptosis and autophagy induced by DHA may be related to mTOR inhibition through AMPK activation and PI3K/Akt inhibition in NSCLC. Therefore, utilization of DHA may represent a potential effective therapy for the chemoprevention and treatment of human non-small cell lung cancer.

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (2011–0013263 and 2011–0006232).

Docosahexaenoic Acid-induced Autophagy is Related to Inhibition of mTor by Reactive Oxygen Species in P53 Mutant Cancer Cells

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Introduction: Our previous studies reported that docosahexaenoic acid (DHA) induces autophagy through p53 inhibition in the wild-type p53 cancer cells. This study attempts to elucidate the molecular mechanism underlying DHA-induced autophagy in PC3 and DU145 prostate cancer cells harboring mutant p53.

Material and Method: Dihydroethidium (DHE) was used for reactive oxygen species (ROS) measurement in cytosol. MitoSox was performed for ROS measurement in mitochondria. Autophagy was detected after docosahexaenoic acid (DHA), an omega 3-PUFA, exposure as indicated by induction of LC3b expression for measuring autophagosome vacuolization. Lipodipomin was used to transfect Akt gene to cells. Apoptosis was detected after docosahexaenoic acid (DHA), an omega 3-PUFA, exposure as indicated by induction of LC3 expression, and formation of autophagic vacuolization.

Results and Discussion: DHA increased both the level of microtubule-associated protein light-chain 3 (LC3b) and the number of autophagic vacuoles in PC3 and DU145 prostate cancer cells harboring mutant p53. Autophagic flux assay confirmed that DHA-induced increase in LC3-II and autophagic vesicles was an outcome of autophagic process activation, indicating that DHA also induces autophagy in p53 mutant cancer cells. DHA treatment also increased the level of reactive oxygen species (ROS) as measured by dihydroethidium staining, and pretreatment of an antioxidant, N-acetylcyesteine (NAC), significantly inhibited the ROS production as well as autophagy induced by DHA, suggesting that ROS regulates the autophagic process triggered by DHA. Further experiments revealed that the ROS-dependent DHA-induced autophagy associated with ROS production was related to a decrease in the activity of mammalian target of rapamycin (mTOR). NAC remarkably restored the decreases in the levels of phospho-mTOR and 4E-BP, an mTOR downstream molecule, induced by DHA as analyzed by the Western blot assay. Furthermore, the level of phospho-AMPK, which negatively regulates mTOR, was increased, while phospho-Akt was reduced during the DHA-induced autophagy, indicating the involvement of AMPK and Akt signalings.
Introduction: One of the most common and biologically aggressive of malignant astrocytic gliomas is glioblastoma (GBM). Studies have revealed several cancer cells growth such as breast cancer cells. Here, we examined the anticancer effect of ω-3 PUFAs in D54 malignant glioma (DSMG) cells. Material and Method: The effects of DHA on cell proliferation and cell cycle were examined by MTT assay and FACS. DHA-induced apoptosis was analyzed using the TUNEL assay, Annexin assay, and western blot. Autophagy was detected after docosahexaenoic acid (DHA), an omega-3 PUFA, exposure as indicated by induction of LC3 expression, and formation of autophagic vacuoles.

Results and Discussion: We show that docosahexaenoic acid (DHA), a ω-3 PUFA, induced apoptosis in DSMG cells, as confirmed by the formation of cleaved PARP, TUNEL assay, and cytofluorometric analysis. DHA-treated DSMG cells also showed a significant increase in autophagic activity as revealed by LC3-II elevation, autophagic vesicles formation and autophagy flux assays, suggesting that both apoptosis and autophagy contribute to the DHA-induced tumor cell death. The DHA-induced cell death in DSMG cells was accompanied by the activation of AMPK and decrease in the Akt phosphorylation. DHA also decreased the activity of mTOR and the level of its downstream molecule 4EBP as analyzed by the Western blot assay. DHA-treated DSMG cells in Fat-1 transgenic mice, that can produce high levels of ω-3 PUFAs, was significantly inhibited, and the apoptotic index was higher compared with wild type mice.

Conclusion: Together, these results suggest that ω-3-PUFAs induce cell cytotoxicity through apoptosis and autophagy in brain cancer. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011–0006232).

Materials and Methods: Radioisensitization was assessed by clonogenic cell survival assay. To investigate the mechanism of PPARγ ligands induced radiosensitization, the subapical cellular DNA fraction was analyzed flow cytometry. Activation of the caspase pathway by combined PPARγ ligands and γ-radiation treatment was detected by immunoblot analysis. To measurement of reactive oxygen species (ROS), the fluorescence of DCF was immediately measured by flow cytometry.

Results: In vitro, these three PPAR gamma ligands enhanced γ-radiation (IR)-induced apoptosis and caspase-3-mediated PARP cleavage. The combined PPAR gamma ligands γ-radiation treatment also triggered caspase-8 activation and this initiator caspase exerted a critical role in the combination-induced apoptosis. In addition, PPAR gamma ligands could also enhanced γ-radiation-induced DNA damage response such as H2AX. Moreover, the combination treatment significantly increased ROS generation, and ROS scavengers NAC inhibited the combined treatment-induced ROS generation and apoptotic cell death.

Conclusions: Taken together, these results indicate that the combined treatment of PPAR gamma ligands and γ-radiation synergistically induced DNA damage and apoptosis, and ROS are critical regulators of these enhanced DNA damage and apoptosis.

Conclusion: Collectively, our results demonstrate that DHA induces autophagy through the ROS-mediated mTOR inactivation in p53 mutant prostate cancer cell line LNCaP. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2011–0006232).

Omega-3 Polysaturated Fatty Acids Inhibit Brain Cancer Cell Growth by Apoptosis and Autophagy

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Materials and Methods: Dynamic remodeling of the actin cytoskeleton underlies increased motile properties of cancer cells. We identify and investigate protein kinases involved in regulating actin cytoskeleton dynamics of MB cells in biased and unbiased approaches. By targeting these kinases, we want to interfere with MB motility and metastatic dissemination. We use in vitro 2D and 3D cell culture systems suitable for advanced microscopy analyses that mimic MB cell dissemination in vivo and have targeted knock down or pharmaceutical inhibition of protein kinases.

Results: In a biased approach, we found that c-Met receptor tyrosine kinase expression and activity are constitutively increased in several MB tumor cell lines and that stimulation with HGF promotes motility and scattering. In MB cells embedded in 3D matrix, activated c-Met receptor accumulates at the invasion front at the leading edge. We also detected increased expression of the levels of CD44 and CD44v6 in tumor-specific MB cell lines. Furthermore, we found that MB cell adhesion promotes c-Met activation independent of HGF and that invasive MB cell behavior requires functional cell-matrix interaction. The implication of other kinases in MB motility regulation will be discussed.

Conclusions: Environmental cues can trigger a motile phenotype in MB. Motility cues are relayed by protein kinases such as c-Met to cellular effectors that mediate dynamic changes in the actin cytoskeleton and drive cell motility. We propose the inhibition of protein kinases controlling MB actin cytoskeleton dynamics and cell motility as a novel approach towards the design of effective treatments for metastatic MB.

Heterogeneity of Cancer-Associated Fibroblasts From Human Primary Colon Tumors in Their Pro-tumorogenic Abilities on Cancer Cells

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Introduction: Cancer associated-fibroblasts (CAFs) constitute the major component of the tumor stroma. CAFs actively participate in reciprocal communication with the tumor cells and with other cell types of the microenvironment, contributing to a tumor-permissive neighborhood and promoting tumor progression. The aim of this study is the characterization of the CAFs from primary human colon tumors regarding their ability to promote tumorigenesis.

Material and Method: Primary CAFs were isolated from 16 primary human colon tumors. After CAFs establishment and growth, fibroblasts were co-cultured with LIM1215 tumor colon cells. Results and Discussion: Co-culture of primary CAFs with LIM1215 cells induced a significant increase in the migration of LIM1215 in a setting in which colon cell were seeded in the upper chamber of trans-well culture system and fibroblasts in the lower one. Interestingly, we could observe significant differences of fibroblasts-derive paracrine pro-migratory effects on cancer cells among CAFs from different patients. In this way, primary CAFs were grouped regarding their ability to promote tumor migration. For further classification, expression of myofibroblasts specific marker and senescence status were analyzed in the primary CAFs. There was an inverse correlation between senescence and myofibroblasts markers. Distinction in the paracrine pro-migratory effects of CAFs was compared with the pathological parameters of primary tumors. Although, no statistical differences were observed, probably due to the low number of samples, there were some remarks to point out. Therefore, CAFs isolated from patients with advanced tumor stages, as well as, those isolated from men presented higher pro-migratory effects. Currently, an inverse association was observed between CAFs pro-migratory potential and histological degree. Future analysis will include gene expression profile, proteomic analysis and secretory phenotype study of these different ‘CAFs promoting migration groups’ to characterize the cross-talk between fibroblasts and colorectal cancer cells. These analyses will allow the search and characterization of new tumor makers.

Conclusion: The CAFs population from the colon tumor microenvironment is heterogeneous regarding different patients presenting different pro-tumorogenic abilities, providing us the chance to search for new cross-talk mediators between fibroblasts and tumor cells that finally could be blocked to inhibit tumor progression.
The HNF4alpha–Cdx2 Axis in the Intestinal Cancer

Identification of Novel Tumor Endothelial Markers in Human Tumour Protein D52

Methods: RNA interference approaches to characterise the effects of reducing TPD52 expression in breast cell lines according to their

levels, while TPD52 protein expression was analysed by western blotting.

Results: 1. LacZ activity and the Cdx2 protein decreased in parallel in the intestinal tumors of pCD2–8 LacZ mice with Apc+/− mice. The

tissue was analysed phenotypically using immunofluorescence analyses and

analyzed independently for TSP2 proteinstaining.

Conclusion: This study demonstrates that the downregulation of HNF4α is an important determinant of the reduced expression of the Cdx2 tumour suppressor gene in intestinal cancers. Consistently, like Cdx2, HNF4α exerts a tumor suppressor function in the colon.

References:


3. Brentani H.P., Diamanti S.M., Belehradek J., Carneiro M., Hemberger M., and Bubendorf L. 2013. TSP2 expression was assessed by immunohistochemistry and RT-qPCR.


7. Brentani H.P., Diamanti S.M., Belehradek J., Carneiro M., Hemberger M., and Bubendorf L. 2013. TSP2 expression was assessed by immunohistochemistry and RT-qPCR.


Introduction: Cdx2 encodes a homeodomain transcription factor that is crucial for gut development and for the homeostasis of the adult intestine. Cdx2 expression is deregulated in colon cancer, becoming heterogeneous or reduced. Our previous data have shown that Cdx2 exerts a tumor suppressor function in the colon, since its downregulation facilitates tumour progression and colon cancer cell migration and dissemination. By analyzing the Cdx2 promoter in transgenic mice and in cell lines, we have previously established that several endodermal transcription factors, including HNF4α and GATA6, are involved in the Cdx2 regulation in the normal gut. Here we have studied the role of HNF4α in the mechanism of Cdx2 deregulation in colon cancers.

Material and Methods: Immunohistochemical analysis of Cdx2, HNF4α and/or GATA6 was performed on intestinal tumors of Apc+/− mice, treated with Azoxymethane (AOM). AOM is a chemical inducer of adenocarcinoma and on human colorectal cancer. The patterns of HNF4α and Cdx2 expression were compared in several cellular models by immunohistochemistry and RT-qPCR.

In vivo, the activity of the 9-kb Cdx2 promoter was analyzed in intestinal transformed cells by crossing pCD2–8 LacZ mice with Apc+/− mice. The

tissue was analyzed phenotypically using immunofluorescence analyses and

analyzed independently for TSP2 proteinstaining.

Conclusion: This study demonstrates that the downregulation of HNF4α is an important determinant of the reduced expression of the Cdx2 tumour suppressor gene in intestinal cancers. Consistently, like Cdx2, HNF4α exerts a tumor suppressor function in the colon.

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7. Brentani H.P., Diamanti S.M., Belehradek J., Carneiro M., Hemberger M., and Bubendorf L. 2013. TSP2 expression was assessed by immunohistochemistry and RT-qPCR.


The importance of angiogenesis in tumour growth and metastasis by supplying nutrients and oxygen is well established. However, the role of endothelial cells as active participants in the tumour environment is poorly understood. To identify factors contributed by the tumor-associated endothelium, we performed immuno-laser capture microdissection (LCM) of blood vascular endothelial cells in conjunction with transcriptional profiling from surgically harvested non-neoplastic and neoplastic tissue of six patients with muscle-invasive bladder cancer (MIBC). We found that most of the previously known markers of angiogenic endothelium showed increased expression in bladder cancer-associated endothelium, and we also identified upregulation of several novel tumour angiogenesis markers. Endocan (endothelial-specific molecule-1) was one of the genes that was highly elevated in tumor-associated blood vascular endothelium. We confirmed this upregulation of endocan both by quantitative real time PCR and immunohistochemical staining. Notably, blood vascular endothelial cells were found to express higher levels of endocan in patients with MIBC (N = 13) compared to patients with noninvasive bladder cancer (N = 36) as shown by immunohistochemical staining. In addition, levels of endocan were significantly elevated in the plasma of patients with MIBC (N = 30) compared to healthy individuals (N = 30). We also found that endocan is upregulated in vitro and in a transgenic in vivo mouse model by VEGF-A. Furthermore, we detected increased VEGF-A concentrations in the plasma of patients with MIBC (N = 40) compared to healthy individuals (N = 30). Endocan silencing in cultured human primary endothelial cells abolished cell migration and tube formation induced by VEGF-A. These data indicate that endocan is an important mediator of VEGF-A-induced angiogenesis and a potential biomarker for MIBC.
Effect of Mesenchymal Stem Cells on Hypoxia-induced Overexpression of Telomere Binding Protein TRF2 Inversely Mutant P53 Gain of Function Via P63

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Background: Hypoxia impairs beta-2-adrenergic receptor (beta-2AR) signaling in a variety of tissues. While hypoxia is a common feature important for the progress of solid tumors, little is known about the effect on beta-2AR signaling in tumor microenvironment. Previously, we reported that systemic administration of mesenchymal stem cells (MSCs) increased engraftment and metastatic colonization of rat osteosarcoma cells.

Material and Methods: In this study, we investigated the effect of MSCs on the hypoxia-induced desensitization of the beta-2AR in the osteosarcoma cells using in vitro and in vivo experimental approach.

Conclusions: The lower TSP2 expression level in MSCs indicates its potential use as a biomarker for the differential diagnosis between PCs and BPH. Additionally, TSP2 protein lower staining pattern in BPH and PCa tissues suggests a possible role for TSP2 in the process of epithelial-stromal interaction. The down regulation of TSP2 in PCs, especially in the stroma compartment, could be a mechanism for tumorigenesis and tumor progression, once TSP2 is related to inhibition of tumor angiogenesis and growth.

Effect of Mesenchymal Stem Cells on Hypoxia-induced Desensitization of Beta-2 Adrenergic Receptors on Rat Osteosarcoma Cells

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Background: Hypoxia impairs beta-2-adrenergic receptor (beta-2AR) signaling in a variety of tissues. While hypoxia is a common feature important for the progress of solid tumors, little is known about the effect on beta-2AR signaling in tumor microenvironment. Previously, we reported that systemic administration of mesenchymal stem cells (MSCs) increased engraftment and metastatic colonization of rat osteosarcoma cells.

Material and Methods: In this study, we investigated the effect of MSCs on the hypoxia-induced desensitization of the beta-2AR in the osteosarcoma cells using in vitro and in vivo experimental approach.

Results: Epinephrine, norepinephrine, and isoproterenol increased cellular proliferation of the rat osteosarcoma cell line COS1NR and MSCS in a dose-dependent and beta-2AR antagonist-sensitive manner. While isoproterenol had significant proliferative effects on MSCs under both normoxic and hypoxic conditions, COS1NR cells did not respond under hypoxic conditions. A sensitivity assay for the beta-2AR revealed that hypoxia impaired the sensitivity of COS1NR cells, but did not affect MSCS. An immunoassay showed no significant change in the expression of hypoxia-inducible factor-1-alpha (HIF1-alpha) in COS1NR cells, but revealed a 15% increase in MSCS after isoproterenol stimulation. However, in COS1NR cells co-cultured with MSCS under hypoxic conditions, isoproterenol caused a significant increase in proliferation, and this effect was inhibited by an anti-interleukin-6 antibody. In rat model, systemic administration of MSCS enhanced the growth of osteosarcoma, and the influence of MSCS was significantly inhibited by interleukin-6 neutralization.

Conclusions: Hypoxia has heterogeneous effects on different components of the osteosarcoma microenvironment. Hypoxia caused a desensitization of the beta-2AR in COS1NR cells alone, but mesenchymal stem cells may still support tumor growth through cellular interactions.

Overexpression of Telomere Binding Protein TRF2 Inversely Related to Telomere Length in Renal Cell Carcinoma

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Background: TRF2 is a telomere binding protein that is exclusively found at the telomeres. This small ubiquitously expressed protein, is estimated to be present at more than 100 copies per chromosome end, binding directly to the tandem array of duplex TTAGGG repeats. The protection of human telomeres crucially depends on this protein and it is reasonable to assume that the relative rates of TTAGGG repeats reflects the need for TRF2 binding. Telomere dysfunction is believed to be the significant factor in carcinogenesis. To elucidate the carcinogenesis mechanism, the expression feature and change in telomere length were investigated in renal cell carcinoma (RCC).

Materials and Methods: Tumor specimens were obtained from 30 patients who underwent nephrectomy for renal cell carcinoma. For comparison, normal renal tissue samples were taken in each case and detected by using immunohistochemical method. Transcriptional expression of TRF2 was estimated by real time PCR. The mean telomere length was determined by southern blotting followed by hybridization.

Results: The expression of TRF2 was significantly higher in the tumor tissue in comparison with normal renal parenchyma. The mean telomere length in RCC tissue was significantly shorter than that in normal renal parenchyma. The mean telomere length in all tissue samples were inversely correlated with the level of TRF2 expression.

Conclusion: Our result suggests that the over expression of TRF2 may work to reduce the telomere length in RCC and could contribute to the carcinogenesis of renal cell carcinoma.

Characterisation of the Tumour-Fibroblast Cross-Talk in a Mouse Model of Disease Progression

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Background: Stromal cells within the tumour microenvironment contribute to tumour progression, and an activated tumour stroma has been associated with poor prognosis in many different cancer types. However, the molecular mechanisms involved in recruitment and activation of stromal cells, and cancer associated fibroblasts (CAFs) in particular, are not well understood. We aim to elucidate the molecular mechanism of CAF recruitment and activation in breast tumours and to define the role of CAFs in disease progression.

Materials and Methods: This study employed the cell lines of the orthotopic 4T1 mammary carcinoma series (4T1, 410.4, 4T07), all of which form primary tumours but differ in their aggressiveness and metastatic potential. To dissect the tumour-CAF cross-talk, the 4T1 series of cell lines were inoculated orthotopically into the 4th mammary fat pad of Balb/c mice. When tumours reached maximum size both tumour cells and CAFs were isolated by FACSorting and whole gene expression analysis was performed on RNA from freshly sorted cells. Bioinformatics analysis was carried out on the expression data, and hits were validated in in vitro and in vivo models.

Results: Marker staining of formalin fixed paraffin embedded tumour sections demonstrated that CAF infiltration and activation correlate with tumour aggressiveness in this model. Wnt7a was identified in the gene expression data as a key tumour cell derived secreted factor up-regulated in the highly aggressive tumours. It was shown to both recruit and activate stromal fibroblasts both in vitro and in vivo. The effects of modulating Wnt7a expression on tumour take, stromal composition and aggressiveness are currently being investigated. Gene ontology and pathway analyses of the CAF expression data implicated CAFs in the modulation of the tumour immune response. Indeed, flow cytometric analysis showed that the immune microenvironment of the highly aggressive tumours is skewed towards tumour tolerance, whereas that of the weakly aggressive tumours towards anti-tumour immunity.

Conclusions: In the studied system CAF recruitment and activation correlates with tumour aggressiveness. Whole genome expression analysis of freshly isolated cells identified components of the tumour-CAF cross-talk in the in vivo setting. These studies provide a basis for identifying stromally involved pathways or processes that can be targeted as part of the treatment regime of invasive cancers.

Mutant P53 Gain of Function Via P63

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Introduction: The transcription factor p53 prevents cells with activated oncogenes or damaged DNA from multiplying by initiating repair or elimination programs and therefore functions as a barrier to tumour development. All tumours have circumvented p53’s control by some mechanism. Roughly fifty percent of cancers express a mutant p53 protein, which no longer activates the tumour-suppressive transcriptional program. In addition to this loss of wild-type function, mutant p53 also gains new oncogenic functions. Mutant p53-expressing mice develop aggressive metastatic carcinomas, which do not occur in a p53-null setting. Importantly, metastasis is the biggest challenge in cancer treatment. Possibly, mutant p53 acquires its new functions by inhibiting the p53-family transcription factors p63 and p73. We have previously shown that mutant p53 can induce the recycling of the epidermal growth factor (EGF)-receptor and a5/1 integrin cell surface receptors, leading to an invasive phenotype. This function depends on the presence of p63, indeed loss of p63 alone is known to promote invasion.

Materials and Results: We are currently investigating, how mutant p53 inhibits p63’s function. Expression of two different hotspot p53 mutants, R175H and R273H in p53-null non-small cell lung carcinoma (H1299) cells induces cellular invasion towards EGF or hepatocyte growth factor (HGF) in an inverted matrigel invasion assay. Furthermore, both R175H and R273H p53 repress p63-induced transcriptional activation in a Luciferase reporter assay under the control of the p63 target gene bullous pemphigoid antigen 1 (BPGP1)-promoter. However, mutant p53 blocks transduction of p63 in R175H p53 binds p63 much stronger than the DNA contact mutant R273H p53, suggesting that the extent of p63 suppression does not reflect the strength of interaction of mutant p53 with p63.
In order to determine whether the p63 suppression of mutant p53 depends on direct interaction of the two proteins, we are narrowing down the interacting domains with the aim of creating a p53 deletion mutant, which does not bind p63 at all. This protein will then be tested for its p63 suppressive and invasive activity.

Conclusion: Any advances we make in understanding how mutant p53 inhibits p63’s function and promotes cell invasion will bring us a step closer to being able to inhibit mutant p53 gain of function in cancers, which would have the benefit of being a tumour-selective therapeutic approach.

The Tumour Suppressor SOX11 is Associated With Improved Cancer-specific Survival Among High Grade Epithelial Ovarian Cancers and is Silenced in Non-expressing in Vitro Models Through Reversible Promoter Methylation

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Background: The purpose of the study was to investigate the correlation of SOX11 expression with survival in epithelial ovarian cancer (EOC), but also to demonstrate that the promoter of SOX11 is silenced in in vitro models by reversible methylation.

Material and Methods: SOX11 expression and clinicopathological data was compared using chi-2 test in a cohort of 154 cases of primary invasive EOC. Kaplan–Meier analysis and the log rank test was applied to evaluate ovarian cancer-specific survival (OCCS) and overall survival (OS) in strata, according to SOX11 expression. Also, the methylation status of the SOX11 promoter was determined by sodium bisulphite sequencing and methylation specific PCR (MSP). Furthermore, the effect of ectopic overexpression of SOX11 on proliferation was studied through [3H]-thymidine incorporation.

Results: SOX11 expression was associated with improved survival of patients with high grade EOC, although not independent of stage. Further analyses of EOC cell lines showed that SOX11 mRNA and protein were expressed in two of five cell lines, correlating with promoter methylation status. Demethylation was successfully performed using 5'-Aza-2'-deoxycytidine (5-Aza-dC) resulting in SOX11 mRNA and protein expression in a previously negative EOC cell line. Furthermore, overexpression of SOX11 in EOC cell lines confirmed the growth regulatory role of SOX11. Conclusions: SOX11 is a functionally associated protein in EOC with prognostic value for high-grade tumours. Reexpression of SOX11 in EOC indicates a potential use of epigenetic drugs to affect cellular growth in SOX11-negative tumours.

Dissecting the Role of the Inhibitor of Differentiation 1 in Breast Cancer Metastasis and Characterization of Pathways Controlling Breast Cancer Stem Cell Phenotype

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Breast cancer is the most common type of cancer in women. There is accumulating evidence that primary breast cancers display marked phenotypic and functional heterogeneity and that only a proportion of cells (tumour initiating cells/TICs) in a breast tumour are capable of tumour initiation, maintenance and metastatic dissemination. Given the current state of technology, direct clinical assessment of TIC eradication is still a distant goal. The major obstacles are identification of new, exclusive TIC markers which will allow us to purify, study and optimize targeted therapies, leading to complete ablation of the tumor.

We have strong preliminary data showing the Inhibitor of Differentiation 1 (Id1) is expressed in rare breast cancer cells with high tumour initiating, self-renewal and metastatic potential (TICs). We have used an Id1 genetic reporter to isolate Id1 expressing cells from the 4T1 syngeneic mouse model of breast cancer. The Id1 positive cells are enriched for tumour initiating, self-renewal and metastasis forming potential both in vitro and in vivo. Knockdown of Id1 using inducible RNAi significantly reduces cell proliferation, tumour growth and metastasis dissemination. We hypothesise that Id1 controls breast cancer metastasis by transcriptional control of the cancer stem cell phenotype.

We are currently profiling the Id1 positive cells as well as cells depleted of Id1 expression to identify molecules and pathways that are required for the breast cancer stem-like cells. We would like to use our model system to identify potential drug targets for metastatic breast cancer.

In Vivo Tracing of Tumor-initiating Cells in Transgenic Mice

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Introduction: Tumors are heterogeneous and contain multiple cell types that have different states of differentiation. It has widely been speculated that only a small number of cells (tumor-initiating cells (TICs)) with stem cell characteristics such as self-renewal, have the ability to maintain full heterogeneity. More differentiated tumor cells, which make up the bulk of the tumor, do not have the capacity to self-renew and differentiate in the variety of cell types found in tumors. Clinically, TICs are of particular interest, since it has been suggested that they may survive therapeutic regimens that generally target fast-dividing cells, and might be the cause for local recurrences. Moreover, it has been speculated that TICs that have spread to distant sites may be responsible for initiating and growing metastases that can even appear many years after surgical resection of a primary tumor. However, at present, the existence of TICs is highly debated since most experiments are based on the transplantation of fac-sorted cells in immune-compromised mice.

Material and Method: In order to trace tumor growth for multiple weeks we use a fluorescent genetic mouse model and intravital microscopy. Our lab has developed several intravital imaging techniques to visualize single cells in living mice over a time span of weeks (e.g. Kedrin et al., Nat Meth 2008; Beerling et al., JCS 2010; Zornig et al., Clin Trans Oncol 2011). We utilize mammary imaging windows and intravital 2-photon imaging in mice that spontaneously develop luminal mammary adenocarcinomas upon the expression of the oncogene PyMT to trace tumor growth.

Results and Discussion: By combining transgenic reporter mice that spontaneously develop breast tumors with the latest intravital microscopy techniques we show that only a small subset of mammary tumor cells (~1 in 100) is able to grow and maintain the mass of the tumor, which shows the existence of TICs that fuel the growth of a tumor. Interestingly, from imaging the outgrowth of tumors over time we noted that the spatial distribution of growth changes over time, which suggests that TICs are highly dynamic and may only temporally reside in a TIC-state.

Conclusion: From our data we conclude that spontaneous breast tumors contain cells that fuel long-term expansion in primary and non-manipulated breast tumors, and that these cells may only temporarily adapt this state.

Proteins and Growth Factors in Colorectal Cancer Pathology and Survival

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Background: Many different biological factors are involved in tumour invasion and metastasis. The levels of key biological factors were determined in colorectal tumour tissue and pre-operative plasma samples and these levels were correlated with tumour pathology and ten year survival.

Material and Methods: 74 pre-operative plasma samples and 101 paired tumour and normal tissue samples were analysed by ELISA for various proteinases, inhibitors and growth factors. In plasma: matrix metalloproteinases (MMP-1, -3, -7), tissue inhibitors (TIMP-1, -2), urokinase plasminogen activator (uPA), PA inhibitor-1 (PAI-1) (ng/ml), and growth factors (pg/ml), vascular endothelial (VEGF), transforming (TGF-β1) and basic fibroblast (bFGF) and in colorectal tissue samples; MMPs-1, -2, -3 and -9, TIMPs -1 and -2, uPA and tissue type PA, PAI-1, PAI-2, the receptor for uPA (uPAR) (ng/ml protein) and VEGF (pg/ml). Levels were correlated with tumour pathology; Dukes' stage, differentiation, tumour depth, vascular and lymphatic invasion (p < 0.05; Spearman's correlation coefficient). A ten year survival analysis was performed (p < 0.05; Kaplan Meier). The study had ethics approval.

Results and Discussion: The tissue levels of all studied proteinases, PAIs and TIMP-1 were all found in significantly greater amounts in the tumour tissue than normal mucosa; e.g. TIMP-1, tumour, median 37.0 (range, 1.5–351.1)ng/mg protein and normal, 11.4 (1.1–107.0). However IPA levels were significantly greater in normal mucosa. Tumour levels of MMPs, uPA, uPAR and PAI-1 significantly correlated with Dukes’ stage. Tumour PAI-1 and uPA levels also correlated with lymphatic invasion, TIMP-1 and PAI-1 with tumour depth and PAI-2 with vascular invasion. Plasma levels of VEGF, PAI-1 and bFGF demonstrated a significant positive correlation and TIMP-2 and uPA a negative correlation with tumour differentiation. VEGF levels also correlated with vascular invasion; tumours that had undergone vascular invasion, median VEGF levels, 211 (range, 14–868) pg/ml and no vascular invasion, 80 (0–785).

Overall survival analysis was found statistically significant differences between plasma levels of both MMP-7 and VEGF, with patients with high levels of these factors having poorer survival. In addition, lower VEGF and bFGF plasma levels were significantly associated with disease free survival.
In tumour tissues a high proportion of active MMP-2 and MMP-9 was associated with poorer disease free and overall survival.

Conclusion: Genetic and biological factors in colorectal tissue and plasma samples significantly correlated with tumour histopathology, disease free and overall ten year survival, in particular plasma VEGF levels.

[155] Dissecting the Role of Runx2 in Mammary Development and Breast Cancer

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Background: The Runx genes are a family of three transcription factors (Runx1, Runx2 and Runx3) known to play essential roles in haematopoiesis, osteogenesis and neurogenesis. In vitro evidence suggests a role for Runx2 in mouse mammary gland development and breast cancer but the definitive in vivo genetic knockout experiment is still lacking due to the early lethality of Runx2 knock-out mice. The aim of this study is to characterize the role of Runx2 in mammary gland development and breast cancer using conditional knock-out transgenic mice.

Materials and Methods: To define the pattern of Runx2 expression in mammary gland development we are using a combination of techniques: qRT-PCR on pure FACS-sorted mammary epithelial cell populations, immunohistochemistry and western blotting. In addition, we are currently investigating the role of Runx2 in mammary stem cells through a combination of primary cell culture and fat pad transplantation assays.

To investigate the role of Runx2 in mammary gland development in vivo we are using loss of function mouse models combining lineage-specific mammary Cre with a conditional Runx2 mouse model. With relevance to breast cancer we are using the well-known mouse model (MMTV-PyMT) and the MDA-MB-231 human breast cancer cell line to investigate the role of Runx2 in the context of tumorigenesis and metastasis.

Results and Discussion: We confirmed that Runx2 is expressed in both mouse and human mammary epithelium. Moreover Runx2 protein levels fluctuate during the mammary developmental stages of pregnancy and lactation to rise again during involution. qRT-PCR analysis on fresh sorted mammary cells indicates that Runx2 is specifically enriched in the basal population which interestingly is the stem cells-enriched mammary compartment. Moreover preliminary results coming from mammophore cultures and 3D colony formation assays seem to indicate an involvement of Runx2 in the mammary stem cell population.

In vivo data from mammary population profiling on the loss of function mouse model indicates that loss of Runx2 is having a direct effect on the mammary epithelium. In particular our data suggest a key role for Runx2 in mammary basal population maintenance, although a milder effect is also detected in the luminal compartment. With relevance to breast cancer preliminary data indicates that Runx2 is expressed in 80% of tumours coming from the MMTV-PyMT model.

Conclusion: Our data indicate that Runx2 is playing a role in mammary epithelial population maintenance, especially in the basal lineage.


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Background: Normal cells divide a finite number of times, although immortalized and cancer cells can proliferate indefinitely and avoid cellular ageing. Cancer transformation is a multistage process mechanisms of which has been studied insufficiently yet. Our work was aimed at studying the processes of cellular ageing, immortalization and transformation of human, mouse and rat embryonic fibroblasts starting from primary cell cultures at the single cell level by computer videomicroscopy.

Material and Methods: Computer videorecordings of living cells during prolonged time intervals were performed.

Results: The man and mouse have great differences in lifespan and the rate of cancer diseases. The causes of these differences are not clear. It was shown that in mouse big cells (up to 8000 \(m^2\)) entered mitoses as frequently as smaller cells. In contrast, in human cell cultures only small cells (less than 2000 \(m^2\)) divided. It is possible that in man there is a relatively more robust, reliable system of blocking mitotic divisions in abnormal (senescent and precancerous) cells. The interrelations between clonal proliferation and abnormalities of mitotic divisions in the immortalized mouse and rat cells were also investigated. 3 three polar mitoses (with three daughter cells) and 7 asymmetric bipolar mitoses which generated two daughter cells of conspicuously different sizes were registered among 71 mitotic divisions in the individual cell lineage. Abnormal mitotic divisions either did not slow the proliferation in cell clones compared with the progenies of cells that divided by normal bipolar mitoses or were followed by the acceleration of divisions in sequential cell generations. Therefore, abnormal mitotic divisions may contribute to the maintenance of the immortalized state of cell populations by means of generating chromosomal instability. Similar stages of cell immortalization and transformation in the human, mouse and rat cell cultures were revealed: intensively proliferating spindle cells constituted an early stage of immortalization, but later on the major part of spindle cells reached their proliferation limit and gradually died, but some spindle cells differentiated into cells with characteristics of mature cells.

Conclusions: Computer videomicroscopy of living cells is a powerful experimental approach for a detailed analysis of stages of cell ageing, immortalization and oncological transformation. For more information, please explore: http://www.labcmp.by.

[158] TrypsinogenIV, a New Target to Impair Tumor Endothelial Cells Motility

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Introduction: Endothelial cells (EC) line the blood vessels and the tumor environment expresses molecules that distinguish it from healthy endothelia. By investigating the transcriptional features of EC isolated from human specimens we have demonstrated the expression of serine protease 3 (PRSS3), a member of the trypsinogen family, by the cancer vasculature. Since proteases play a pivotal role in angiogenesis, the aim of this study was to characterize PRSS3 expression by EC and its possible involvement in the angiogenic process.

Material and Methods: EC were isolated from ovarian carcinoma (HOC-EC) and normal tissues (human adrenal gland and skin/derma) and cultured in vitro. Gene expression was examined by RT-qPCR. Motility and proliferation were assayed by 'wound-healing' and MTS assay, respectively. PRSS3 silencing was achieved by small interfering RNA (siRNA).

Results and Discussion: We established that EC express TrypsinogenIV, as the splice variant of PRSS3 transcript, and do not express PRSS1 and PRSS2, the most studied members of the trypsinogen family. EC were assayed in culture conditions mimicking either the 'non-neoplastic' environment surrounding normal-EC in healthy tissues or the 'tumor pro-angiogenic' environment in which the tumor-EC are embedded. We demonstrated that both HOC-EC and normal tissue derived EC up-regulate TrypsinogenIV in response to a pro-angiogenic milieu (i.e. VEGF-A, FGF-2 and EGF, known to be abundant in the tumor microenvironment). However, the amount of transcript is much higher in HOC-EC than in normal tissues derived EC (regardless of the pro-angiogenic condition). These findings suggest that EC can modify their phenotype in response to the cancer-provided stimuli and as a consequence initiate and sustain tumor angiogenesis and vascular remodeling through TrypsinogenIV. Indeed, HOC-EC migration and proliferation is enhanced by a pro-angiogenic milieu. TrypsinogenIV depletion by siRNA - profoundly impaired HOC-EC capability to close the wound (whereas we saw no effects on proliferation). Thus, TrypsinogenIV induction results essential for EC motility in response to the tumor environment.

Conclusion: The present study provides evidence that PRSS3/TrypsinogenIV is the trypsinogen member expressed by EC and uncovers a previously unrecognized role for Trypsinogen IV in the angiogenic process and tumor vessel remodeling. Supported by the Italian Association for Cancer Research (AIRC) and the CARIPLO foundation.

[159] The Role of the Biophysical Properties of the Glycocalyx and Extracellular Matrix Tension in Enhancing Glioma Cell Invasion

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Background: Adult brain cancer continues to be a significant challenge to both clinicians and patients with more than 20,000 new cases diagnosed yearly and incredibly poor mean survival of 12–14 months for individuals with glioblastoma, the most common type of adult brain cancer. The mortality associated with late stage GBMs can be largely attributed to their early dissemination and intrinsic death resistance. High grade GBMs are notoriously resistant to therapy and typically disseminate throughout the brain tissue, severely compromising patient treatment. Brain cancer environment is highly vascular, abundant in hyaluronic acid (HA) and proteoglycans/linker proteins that are deposited by the tumor cells which mechanically reinforce the extracellular matrix and locally elevate the interstitial pressure from fluid build-up. Thus, the microenvironment of the most aggressive GBMs can be viewed...
as mechanically-challenged and the cells themselves are likely to have altered mechanosensitivity.

**Materials and Methods:** Traction force microscopy (TFM) was employed to measure myosin-dependent cell contractility. Atomic force microscopy (AFM) was used to measure cellular and tissue rheologies. Polyacrylamide gels of varying stiffness were employed to measure mechanoresponsiveness of low-grade and aggressive GBMs by analyzing their cell spread area, survival, and proliferation as a function of stiffness.

**Results:** We determined that high grade GBM cells are softer than the less aggressive low grade gliomas. AFM measurements of freshly excised murine brain tumor tissue slices showed that GBMs extracellular matrix stiffness is higher than that of non-malignant controls. Preliminary findings also showed that isolated high grade GBM cells but not low grade oligodendroglioma cells have a greatly enhanced mechanoresponsiveness e.g., they spread more on soft gels and possess a vastly altered glycolcylx, traits that predict elevated contractility and integrin signaling, which were also quantified.

**Conclusions:** We have shown that GBM aggressiveness likely arises, at least partially, due to alterations in cellular compliance and HA-induced changes in the glycocalyx which drives integrin clustering and alters cell adhesion. Thereby, aggressive GBMs have unique physical properties which, if found to be robust, can be used as means for identification of the cancer-initiating and propagating cells within tumor masses from the cells lacking the aggressive and metastatic potential.

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**Poster Sessions**

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**Investigation of TLR7/8 and 9 Agonists and CD40-ligand Costimulation on EBV Transformation of B Cells from Chronic Lymphocytic Leukemia**

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**Background:** In vitro EBV infection of normal B cells induces early polyclonal activation and subsequent transformation of a small proportion of infected cells. EBV transformation leads to immortalization and establishment of lymphoblastoid cell lines (LCLs). Leukemic B cells of chronic lymphocytic leukemia (CLL) are refractory to EBV transformation. We have recently shown that costimulation with TLR7/8, 9 agonists and to a lesser extent CD40 ligand (CD40L) enhances the transformation efficiency of normal B cells by EBV.

**Material and Methods:** In this study the stimulatory effects of TLR7/8 (R848), TLR9 (Cpg) agonists and CD40L on efficiency of transformation of peripheral leukemic B cells from nine CLL patients were investigated. The effect of these stimulants on proliferation of EBV infected cells was studied by radioactive thymidine incorporation. Furthermore, the leukemic origin of transformed cells was determined by flow cytometry, RT-PCR and sequencing of the immunoglobulin heavy chain variable region (IGHV) genes.

**Results:** R848 and Cpg induced a significant proliferation in CLL B cells (p < 0.001). While EBV alone did not induce substantial proliferation, costimulation with Cpg or R848 boosted the proliferative response and also increased the frequency of transformation of EBV infected CLL B cells (p < 0.001). Nevertheless, none of the stable LCLs established from the patients was originated from the leukemic B cells of chronic lymphocytic leukemia (CLL) and IGHV gene sequence profiles. One LCL was found to counteract the pro-transcriptional effect of HMG1A on the TLR7/8 promoter. Expression analyses performed on human carcinomas of different anatomic origin and different degree of malignancy showed an inverse correlation between CBX7 and SP/1/HMG1A gene.

**Conclusion:** Our data show that CBX7 may be able to prevent cancer progression, by repressing the SP1 gene, through a mechanism involving the interaction with HMG1A protein on this promoter.

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**Identification of Metastasis-associated Glycoproteins in Colorectal Cancer**

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**Introduction:** Cancer metastasis is a multi-step process involving alterations in protein glycosylation. The lectin from Helix pomatia (HPA) has been shown to identify glycoconjugation changes associated with metastatic phenotype, however the glycoconjugates that bind to the lectin have yet to be characterized in detail. It is recognised that HPA binding partners could prove useful as drug targets or biomarkers for cancer metastasis. We carried out a proteomic study to identify the HPA binding glycoproteins in colorectal cancer tissue samples.

**Materials and Methods:** Tumour and matched healthy tissues were used (metastatic n = 6 and non-metastatic n = 5). The glycoproteins were enriched on an HPA affinity chromatography column and separated using one- and two-dimensional gel electrophoresis and identified using mass spectrometry. Verification of the proteins detected was carried out using Western blotting. The correlation between HPA binding and PS3 levels was investigated using a univariate linear regression, the degree and significance of variation in carcinoembryonic antigen (CEA) expression between lymph node positive and negative patient samples was investigated using anti-CEA monoclonal antibodies.
Results and Discussion: A comparative analysis of the HPA binding of proteins from the two sample groups showed greater levels of HPA binding proteins in the metastatic samples, reflecting an increased expression of GaINAc containing proteins including Tn and siaI-Tn antigen in metastatic cancer. Annexin IV/V and calcium-activated chloride channel protein 1 have been identified as up-regulated HPA binding partners expressed in the metastatic sample group. There was a positive correlation between HPA binding and CEA expression profiles but no clear relationship between HPA staining and abnormal PS3 staining.

The precise role of HPA binding partners in metastatic colorectal cancer has yet to be understood, this study confirmed the prognostic utility of HPA for metastatic colorectal cancer. Further work is necessary to obtain more detailed mapping of the HPA binding proteins in order to reveal useful biomarkers for metastatic colorectal cancers. The next step will be analysis of a larger, more diverse, patient population to enable validation of these candidate tumour and bio-markers.

[155] Influence of HOXA9 Expression in the Response of Glioblastoma Cells to Temozolomide

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Introduction: Glioblastoma (GBM) is simultaneously the most common and most malignant (WHO grade IV) subtype of glioma, exhibiting remarkable resistance to treatment. Recent studies showed that the overexpression of HOXA9, an important transcription factor during development, is associated with a worse prognosis of patients with GBM. In addition, HOXA9 has been shown to increase cell viability and reduce cell death. Because GBM patients are standardly treated with Temozolomide (TMZ), an alkylating chemotherapeutic agent, we hypothesized that HOXA9-overexpressing cells would be more resistant to TMZ, which may partly explain its association with poor prognosis.

Materials and Methods: To assess the influence of HOXA9 in the response to TMZ, we used an established human GBM cell line (U87-MG) and immortalized human astrocytes (hTERT/E6E7) as putative glioblastoma precursor cells. Since both cell lines do not endogenously express HOXA9, they have been previously genetically engineered with retroviral vectors to obtain stable HOXA9-overexpressing cells. The effect of HOXA9 expression in the cellular response to TMZ was measured at the levels of cellular viability, cell death and cell invasion. Half maximal inhibitory concentration (IC50) was calculated for each cell line. Cell viability was assessed by trypan blue and MTS assays, cell death by annexin V and propidium iodide staining and cell invasion was performed by matrigel invasion assay. Finally alteration on DNA repair mechanisms was measured by western blot. We evaluated stemness capacity by neurospheres forming assay and dilution assay.

Results: HOXA9-overexpressing cells showed higher IC50 values when compared with HOXA9-negative cells, both in U87-MG and hTERT/E6E7 cells. Cell viability was higher in cell lines that expressed HOXA9, both in basal conditions and after treatment with different doses of TMZ. TMZ-induced cell death was observed both in U87-MG and hTERT/E6E7 cells, but this was less prominent in cells overexpressing HOXA9. Additionally, HOXA9-overexpressing cells showed higher invasion capacity under basal and treatment conditions.

Conclusion: Our results suggest that overexpression of HOXA9 renders cells more resistant to TMZ, which might partly explain its association with poor prognosis of GBM patients. These findings may have clinical significance in the future, allowing a better prediction of patients’ response to chemotherapy and assisting the clinicians in therapy choices.

[160] The Cellular Effects of the Oncogene Pp32r1 (ANP32C) and Its Functional Mutant

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Background: The pp32 gene acts as a tumor suppressor while its closely related homologue pp32r1 is oncogenic and has been shown to be over expressed in tumors of the breast, prostate and pancreas. A naturally occurring pp32r1 mutant (p. Tyr140His) has been previously identified in prostate cancer cells, that results in a phenotype illustrating a very high proliferative capacity when transfected into other cells. However, little is known about the cellular localization and mechanism underlying the oncogenic potential of pp32r1 and pp32r1-1H.

Methods and Materials: ACHN kidney cancer cells were transfected with self inactivating (Sin) lentiviruses carrying pp32r1 and pp32r1-Y140H mutant genes. On these cell lines we performed confocal microscopy (Olympus TX81) using anti-pp32r1 antibody.

Cell cycle analysis, BrdU incorporation assay, CHD4 siRNA knockdown, MTS cell viability assay and Annexin V-Apoptosis assay and isotope-coded protein labelling Mass Spectrometry.

Results: We first generated lentiviral transduced ACHN cell lines over expressing pp32r1 and the mutant form and demonstrated by comparative Cell cycle analysis and flow cytometry that, the enhanced proliferation rate in the cells expressing pp32r1/Y140H is largely due to an influence on the G1 to S phase of the cell cycle.

We also used these HOXA9 as an example to illustrate by confocal microscopy that pp32r1 and pp32r1-Y140H reside predominantly in the cytoplasm unlike pp32r1, which is a known nuclear/cytoplasmic protein.

A comprehensive MS-derived proteome analysis of ACHN, ACHN-pp32r1 and ACHN-pp32r1/Y140H cell lysates using the isotope-coded protein label (ICPL) method identified a number of differentially regulated proteins. We confirmed by real time and western blotting that Chromodomain Helicase DNA binding protein 4 (CHD4 – promoter of DNA double strand break repair and cell survival) was significantly up regulated at the protein level. CHD4 knockdown studies have further confirmed its significance and influence on the cell cycle.

Pharmacological relevance of the up-regulated expression of pp32r1 and pp32r1 mutant in cancers has been demonstrated by its ability to offer an increased apoptotic resistance to fingolimod (FTY720), a potent apoptosis inducer and potential chemotherapeutic drug. From in vitro Pull down studies using recombinant proteins we have observed that both pp32r1 and pp32r1 mutant could bind fingolimod (FTY720). Cell viability and apoptosis experiments confirmed a reduced susceptibility to the drug in cell lines over expressing pp32r1 or pp32r1 mutant.

Conclusion: From our data, we have observed a cytoplasmic localisation of pp32r1 and pp32r1 mutant. Our studies have attributed the enhanced proliferative phenotype to a G1 to S phase influence of pp32r1 mutant, mediated through CHD4 up regulation. Further, we have shown differential susceptibility of cell lines over expressing pp32r1 and pp32r1 mutant to the potential chemotherapeutic drug fingolimod (FTY720).

[167] Functional Inhibition of Ubiquitin Conjugating Enzyme E2C Sensitizes Breast Cancer Cells to Radiation and Chemotherapy Drug

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Introduction: The incidence of breast cancer is rising in every country of the world especially in developing countries such as India. According to the Madras Metropolitan Tumor Registry, breast cancer is the leading cancer in women and has overtaken carcinoma cervix with crude incidence rate of 30/100000. Ubiquitin-conjugating enzyme E2C (UBE2C) is a member of proteasome system, which is responsible for ubiquitination of mitotic cyclins. Overexpression of UBE2C was reported in lung, gastric, bladder, and uterine cancer. The objective of the study was to elucidate the functional capabilities of UBE2C, to explore the potential of UBE2C as an extracellular marker and therapeutic target in breast cancer.

Material and Methods: Breast cancer cell lines MCF-7, BT-549, MDMAB-231, T47D and a normal cell line HEK293T were used in the study. Taqman Real time PCR was done for UBE2C levels in breast cancer cell lines and HEK293 cell line. Inhibition and expression levels of breast cancer cells were analyzed by MTS assay. For functional characterization a dominant negative mutant of UBE2C (DN-UBE2C) was transfected in MCF-7 and MDMAB-231 cells. Growth curve, radiation and chemo response of DN-UBE2C transfected cell lines were assessed by MTS assay and colonogenic assay. Cell cycle analysis was done by FACS. For statistical significant p-value of <0.05 was considered significant.

Result and Discussion: Among the cell lines analyzed, copy number of UBE2C mRNA transcripts was found to be highest in MDMAB-231 (four fold) followed by about two fold upregulation in T47D, BT-549, whereas MCF-7 showed least expression compared to relative expression HEK-293T cells. Radiation and chemotherapy drug response of the breast cancer cell lines revealed, at 8 Gy of radiation dose MDMAB-231 was found to be most resistant cell line whereas BT-549 cells were most sensitive. MCF-7 was most resistant to Adriamycin but found to be most sensitive to 5-fluorouracil (5-FU). To confirm the expression levels of UBE2C with radiation response, MDMAB-231 cells expressing fourfold overexpression of UBE2C was found to be the most resistant cell line. However, MCF-7 cells which has lowest level is only moderately sensitive and BT-549 cell line with two fold upregulation of UBE2C was observed to be most sensitive undergoing the breast cancer cell lysis.

The growth rate and anchorage independent growth of MCF-7 and MDMAB-231 transfected with DN-UBE2C was significantly reduced compared to cells transfected with vector alone. Cell cycle analysis was also observed in MCF-7 and MDMAB-231 transfected with DN-UBE2C compared to cells transfected with vector alone. Significant decrease in resistance to radiation and to Adriamycin by MCF-7 and MDMAB-231 cells transfected with DN-UBE2C was observed when compared to vector alone transfected controls. This effect might possibly due to competitive inhibition of functional wild type UBE2C. However, the precise molecular mechanisms by which DN-UBE2C sensitizes cells to radiation and Adriamycin are unclear and needs further investigation.
**Conclusion:** This study correlates the expression of UBE2C to radio and chemo resistance of breast cancer cells, providing a potential target in a subset of patients whose tumors express high levels of UBE2C in breast cancer.

**Material and Method:** A conditional knock-in, Cre-lox-regulated mouse model (Braf^LSL^F597V) was used to express the L597V Braf mutation in mouse tissues and embryonic fibroblasts (MEFs). To examine cooperation between L597V Braf and oncogenic RAS, Braf^LSL^F597V/Braf^LSL^G12D mice were intercrossed with the Jacks/Tuveson Kras^LSL^G12D conditional knockin mice. The effects of the mutations on cancer hallmarks and downstream signalling pathway activation were examined.

**Results and Discussion:** Constitutive expression of endogenous L597V Braf induced Braf activity by ~2-fold and led to weak activation of the downstream Mek/Erk pathway. This was associated with induction of RASopathy hallmarks including facial dysmorphia, short stature and cardiac hypertrophy but was not sufficient to transform MEFs in vitro or induce tumours in vivo. By co-expressing V500E Braf with oncogenic G12D Kras, the two mutations synergised to increase Mek/Erk signalling to levels comparable to that induced by the high activity mutant V600E Braf. Morphological transformation of MEFs was more similar to that induced by V600E Braf than G12D Kras and microarray analysis confirmed that the double mutant cells had a gene expression signature more similar to V600E Braf than G12D Kras. In the lung, there was also a shift from predominantly adenomatous alveolar hyperplasia lesions, normally induced by G12D Kras, to predominantly adenomas with some predisposition to cancer.

**Background:** Thyroid carcinoma arising from the thyroid follicular epithelium represents the most frequent endocrine malignancy and is mainly associated with activating RET/PTC, NTRK3 and BRAF mutations. BRAF^V600E and RAS^V12 activating point mutations. Except for p53, which appears to be involved only in poorly differentiated and aggressive histotypes, a role of tumour-suppressor genes in the pathogenesis of thyroid cancer is still poorly known.

**Materials and Methods:** Papillary thyroid cancer (PTC)-derived TPC1 and anaplastic thyroid cancer (ATC)-derived FRO cell lines stably expressing pEGFP-C2 empty vector or pEGFP-PATZ1 plasmid, were generated. In these cell lines we performed different functional assays (i.e. growth curves, FACS, colony forming, TUNEL, wound healing, trans-well migration, soft agar growth and xenograft in nude mice) to characterize differences in growth, death, migration, invasive capacity and malignancy, with or without PATZ1.

**Results:** Restoration of PATZ1 expression had no effect on the growth of both cell lines. Conversely, it led FRO cells to apoptosis and highly reduced the migratory and invasive capabilities of both TPC1 and FRO cells. Finally, differently from the FRO parental cell lines expressing the empty vector, the PATZ1 transfectants did not form colonies in soft agar and had reduced transforming ability in vivo compared to their controls.

**Conclusions:** Our data suggest that downregulation of PATZ1 expression exerts a functional role in the pathogenesis of thyroid cancer, and are also consistent with a specific role of PATZ1 in the signaling pathways involved in cell survival and metastatic progression in both papillary and anaplastic thyroid cancer.

**Bcl-2 Family Members and Survival Under Stress Conditions in Multidrug Resistant Leukemic Cells**

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Over-expression of several anti-apoptotic Bcl-2 family members has been reported in hematologic malignancies and has been associated with survival and/or chemoresistance in various human cancers. Thus, differential expression of a single Bcl-2 family member can disturb the balance between life and death and the permutations for pro-survival signaling in the context of cancer cells. In an attempt to address this question in leukemic cells we have investigated the Bcl-2 family expression profile in MDR leukemic cells (L1210/R) compared with its parental cell line, L1210 and with P-gp-overexpressed transfected parental cells (CBMC-6). We have undertaken the study comparing two different kinds of stress conditions: cold temperature and daunomycin exposure. Cold temperature induces preferential cell death in leukemic MDR cells while daunomycin treatment induces cell death in parental but not in sensitive cells. We have focus here in the study of Bcl-xL and Bcl-2, as anti-apoptotic members; and Bax (belonging to the multi-domain or Bax subfamily) and Bad (belonging to the Bcl-x only subfamily) as pro-apoptotic members. By using western-blot techniques, we have detected expression levels of Bcl-2 family members in leukemic cells treated with daunomycin for different doses and/or time points. Furthermore, we have performed silencing experiments in order to analyze the contribution of each individual member of the Bcl-2 family to the survival of parental (L1210), resistant (L1210R) and P-gp-transfected (CBMC-6) leukemic cells in presence of the chemotherapeutic agent. We have compared these data with those obtained previously by using the model of cold-stress-induced cell death. Together, these findings demonstrate that during the process of drug resistance, leukemic cells undergo alterations on Bcl-2 family members expression that could influence their response to different types of stress.
Expression of Apoptosis and Cell-cycle Regulators in Rat Prostate Overexpressing Reguculin

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Background: Reguculin (RGN) is a calcium (Ca2+)–binding protein, also known as S100 protein-30 (SMP30) by its characteristic down-regulated expression along with aging process. RGN plays a role as Ca2+–homeostasis regulator and has been shown to catalyse an important step in L-aspartic acid biosynthesis being also associated with protection against oxidative stress. In addition, in vitro overexpression studies have been indicating that RGN has suppressive effects on cell proliferation and apoptosis. Moreover, hepatocyes from SMP30 knockout mice are highly susceptible to tumor necrosis factor-α and actinomycin D induced-apoptosis. Recently, we reported a diminished expression of RGN in human prostate cancer cases which correlates with cellular differentiation of prostate adenocarcinoma. In the present study we analyzed the expression of cell-cycle and apoptosis regulators in the prostate of transgenic rats overexpressing RGN in comparison with their wild-type counterparts.

Material and Methods: Sprague Dawley rats overexpressing RGN (Tg-RGN) were obtained from Japan SLC, Inc. Whole prostates (n=7 for each group) were collected from Tg-RGN and wild-type 3 month-old animals, and longitudinally divided for mRNA and protein extraction. Expression of cell-cycle and apoptosis regulators was determined by means of real-time PCR and Western Blot.

Results and Discussion: Tg-RGN rats showed altered prostatic expression of Bcl-2, BAX, caspase 9 and caspase 3, when compared with wild-type animals. Also, the expression of oncoproteins, H-ras and c-myc, and that of tumor suppressor gene p53 was modified in Tg-RGN rats. Chk2 and p21 expression was not significantly changed in the prostate of Tg-RGN rats. Cellular homeostasis is maintained by the establishment of a tight equilibrium between cell death, survival and proliferation. The altered patterns of above mentioned proteins in the prostate of animals overexpressing RGN, suggest it may play a role in the control of apoptosis and proliferation of prostate cells. Conclusion: RGN seems to play a role in cell death/survival balance of prostate cells and therefore may be involved in prostate tumor development and/or progression.

A Novel MUC16 (CA125) Monoclonal Antibody

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Introduction: The MUC16 mucin was identified as the serum antigen detected in the CA125 biomarker assay to monitor patients with ovarian cancer. A number of monoclonal antibodies (MAbs) including OC125 and M11 are available to detect MUC16. Despite considerable efforts the epitopes detected by these MAbs have remained elusive and glycosylation has been proposed to play a role for the epitopes. Existing MAbs react with MUC16 expressed in both normal and cancer cells and hence detect enhanced levels of MUC16 derived from both benign and malignant cells.

Material and Methods: In this study, we used an E. coli expressed MUC16 fragment glycosylated in vitro with Thr for immunization of mice and selected a novel MAb (5E11) with a cancer reactivity. Overlapping peptides covering the tandem repeat domain were used for epitope mapping in ELISA and microarrays assays.

Results and Discussion: Comprehensive analysis of the fine specificities of existing MUC16 MAbs and the new MAb have been undertaken and we found that existing MAbs react with a conformational epitope of the 156 amino acid tandem repeat sequence of MUC16 without dependence of O-glycosylation. The novel MAb in contrast reacts with a linear peptide epitope, which expression is dependent on glycosylation.

Conclusion: We characterized existing MAbs for MUC16 (M11 and OC125) and produced a new MUC16 MAb (5E11) that recognizes a glycosylation dependent epitope on the tandem repeat region of MUC16.

Quantification of 3D Tumor Vessel Networks by Vascular Caliber, Density, Lumen Diameter, Allows Comparison Among Effects of Vascular-targeted Therapies

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Background: Assessment of the efficiency of anti-tumor, vascular-targeted therapies is hampered by difficulties in objective quantification. In this respect, we speculated that maps of very thin capillaries might be fingerprints of a given vascular tree. To test this hypothesis, we analysed whether amount and distribution of capillaries differed by caliper might be a characteristic feature in tumors treated with antiangiogenic or antivascular drugs.

Material and Methods: In vivo experiments were performed in NOD/SCID mice bearing subcutaneous KMS-11 human tumors treated, or not, with sorafenib, sunitinib and combretastatin-4-phosphate (CA4P). In vivo biolivation of endothelial cells with sult-NHS-LC-biotin allowed imaging of functional vessel networks at high magnification (40x), leading to 3D reconstruction of tumor vasculature. Custom ImageJ routines were used to classify vessels, and to quantify signal amounts and distribution, extending to 3D a planar space-filling approach (Righi et al., Lab. Invest. 89, 1063–1070, 2009).

Results: Treatment-specific changes were observed after vessel classification according to approximated cross-section (up to 10 μm in diameter). These results highlighted, in all samples, an unexpected relationship between microvessel amounts and distribution. Vessel skeletonization minimized the artefacts due to drug-specific wall swelling and allowed unbiased comparisons. All treatments reduced vessel sprouts up to calibers of 2.5 μm but only antiangiogenic drugs markedly reduced the signal from larger vessels up to 2–4 folds. In addition, CA4P did not alter the spatial distribution of vessels, whereas antiangiogenic drugs caused an increasing clustering, according to vessel diameter. Finally, we analyzed the relationship among the spatial distributions of microvessels from contiguous classes as an indirect indicator of vessel branching. Again, CAAP gave results similar to untreated tumors whereas results from both antiangiogenic drugs diverged significantly, suggesting a drop in branching in the analyzed networks. Thus, our data appear in accordance with what is known about the effects of the analyzed drugs.

Conclusions: In this work we report that quantification of parameters of very thin microvessels can provide a way to characterize pathological vascular trees. We propose this approach as an integrated, yet multifaceted, analysis to quantitate the effects of vascular-targeted therapies.

MYC Directs Transcription of MCL1 and EIF4E Genes to Control Sensitivity of Gastric Cancer Cells Towards HDAC Inhibitors

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Introduction: Histone deacetylases (HDACs) are providing fine tuned epigenetic regulation of numerous essential processes in cells. Deregulated expression of HDACs is observed in many cancers and therefore HDAC inhibitors (HDAI) are currently investigated in clinical trials. However, mechanisms controlling the responsiveness of cancer cells towards HDAC inhibitors are incompletely understood.

Material and Method: In order to investigate resistance toward HDACi we used eight human and murine gastric cancer cell lines and treated them with suberylanilide hydroxamic acid (SAHA). IC50 values were calculated from MTT viability assays and apoptosis levels were determined using PI/Annexin V staining and fluorescence activated cell sorting (FACS). Protein and mRNA levels were investigated by western blot and qPCR, respectively. Molecules conferring HDACi resistance to gastric cancer cells were targeted pharmacologically and by RNA interference. Promoter analysis was conducted by quantitative ChIP assays.

Results and Discussion: Gastric cancer cell lines resistant towards SAHA treatment had higher protein levels of anti-apoptotic Bcl2 family members, Bcl-xl and Mcl1. siRNA mediated knockdown of Mcl1 and Bcl-xl increased sensitivity of gastric cancer cell lines for SAHA. It is known that c-myc overexpression is associated with therapeutic resistance of certain tumors. Consistently, pharmacologically as well as genetically inhibition of c-myc increased sensitivity towards SAHA. Interestingly, c-myc drives the expression of both anti-apoptotic Bcl2 family members, Bcl-xl and Mcl1. Whereas the Mcl1 gene promoter is directly regulated by c-myc in gastric cancer cells, Bcl-xl is indirectly controlled by c-myc. We observed that c-myc binds to the promoter of the elf4e gene, a rate-limiting factor of eukaryotic translation. Knockdown of elf4e decreased expression of Bcl-xl, suggesting that the c-myc/elf4e axis controls translation of Bcl-xl.

Conclusion: Our data reveal a new molecular mechanism for how c-myc controls SAHA responsiveness of gastric cancer cells and provide a rationale for a concerted inhibition of HDACs and c-myc in gastric cancer.
Roles of PDCD4 and eIF4A1 in Breast Cancer

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Introduction: Programmed Cell Death 4 (PDCD4) is a tumour suppressor protein that is important in translational control. It binds and inhibits eukaryotic initiation Factor 4A1 (eIF4A1), a helicase unwinding 5’UTR secondary structures. PDCD4 appears to function in different ways depending on cell type and genetic background and, therefore, it is important to study its role in a context-dependent manner. This project focuses on understanding global and message-specific translation regulation by PDCD4 and eIF4A1, and phenotypes induced by those interactions in breast cancer.

Material and Method: Immunohistochemical analysis of PDCD4 and eIF4A1 expression was performed on a set of 3,605 cases of archival breast tumours (SEARCH). Transient knock-downs of PDCD4 and eIF4A1 were obtained using siRNA. Polysome fractionation was performed on sucrose gradients, and total, subpolysomal and polysomal RNA was collected from control, PDCD4 and eIF4A1 knock-down cells, and analysed on gene expression microarrays, followed by differential gene expression and pathway enrichment analysis. PDCD4 and eIF4A1 levels were assessed by Western blotting. Stable knock-downs of PDCD4 and eIF4A1 knock-downs of cells were generated using lentiviral infections, and growth and migratory properties were assessed.

Results and Discussion: In the patient cohort studied, PDCD4 expression is strongly predictive of survival in estrogen receptor positive breast cancer patients. PDCD4 resides in both nuclear and cytoplasmic compartments. Nuclear PDCD4 correlates negatively with grade, stage, tumour size and lymph node status, and the proliferation marker Ki67. It positively correlates with the Luminal A tumour subtype and Bc2-2. In contrast, eIF4A1 is predictive of poor outcome in estrogen receptor negative tumours. PDCD4 levels differ across breast cancer cell lines and increase with confluency. Transient knock-down of PDCD4 in MCF-7 exerts few phenotypic effects, whereas knock-down of eIF4A1 causes morphological changes and decrease in cell growth. Stable PDCD4 over-expression results in slower growth phenotype. Transient PDCD4 knock-down yields very few differentially expressed genes, consistent with the very high levels of eIF4A1 expressed by this cell line. In contrast, eIF4A1 knock-down has a very pronounced effect on the polysome profile and affects many genes involved in cell adhesion, cytoskeleton remodelling, apoptosis, development, cell cycle, translation and metabolism.

Conclusion: Here we show that PDCD4 and eIF4A1 can differentially predict survival depending on breast cancer subtype. Moreover, knock-downs of these molecules in MCF7 affect the expression of different sets of genes, while only eIF4A1 knock-down induces an obvious phenotype in cell culture. We propose a model to show how dysregulation of these key translational regulators contributes to the malignant phenotype in breast cancer.

Effect of Androgens on the Expression of Ca2+-binding Protein, Regulation of STEAP1 expression in MCF-7 Cells

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Background: Androgens are the precursors hormones, but in addition to their role as precursors hormones, have also been suggested as important agents in breast physiology. However, the precise function of androgens on breast cancer development requires the elimination of key tumor suppressors, and their role as precursors hormones, have also been suggested as important agents in breast physiology. However, the precise function of androgens on breast cancer development has not been fully elucidated. Ca2+ is a ubiquitous second messenger that controls mostly of cell signaling pathways, and altered Ca2+ homeostasis has been associated with development of various types of cancer. There are evidences that STEAP1 may be a useful marker for several types of cancer with different doses of DHT or E2 for several periods of time. The possible signaling pathways were accessed by exposing LNCaP cells to flutamide, ICI and cycloheximide. In vivo, 3 months old wistar rats were divided into 4 distinct groups that included orchidectomised animals treated with either DHT or vehicle, and intact animals treated with DHT or vehicle alone. STEAP1 expression in each experimental group was carried out by Real-time PCR and Western blot. Immunohistochemistry was performed in tissue microarrays (TMAs) of human prostate cancer and non-neoplastic lesions. Correlation between STEAP1 immunoreactivity and clinical data was established using a SPSS program.

Results and Discussion: STEAP1 expression is inhibited by the presence of DHT and E2 in LNCaP cells. The effect of DHT is time- and dose-independent, but E2 appears to trigger STEAP1 down-regulation only after 24h at all concentrations. The signaling pathways by which DHT decrease STEAP1 expression seems to involve the activation of AR. In vivo, castration of adult rats increases STEAP1 protein expression when compared to intact rats, and treatment with DHT abrogates the effect of castration in STEAP1 expression. However, these effects were not observed at STEAP1 mRNA level, suggesting that mechanisms underlying the regulation of translation may be involved. E2-treatment has no effect on Ca2+-channels expression in MCF-7 cells, suggesting that androgen’s role in breast pathophysiology is linked to the control of intracellular Ca2+ homeostasis.

6EAP Ubiquitin Ligase Regulates PML-induced Senescence in Myc-driven Lymphomagenesis

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Neoplastic transformation requires the elimination of key tumor suppressors, which may result from E3 ligase-mediated proteasomal degradation. We previously demonstrated a key role for E3 ubiquitin ligase E6AP (E6-associated protein) in the regulation of PML (promyelocytic leukemia protein) stability and formation of PML nuclear bodies. Here, we report the involvement of the E6AP-PML axis in B-cell lymphoma. A partial loss of E6AP attenuates Myc-induced B-cell lymphomagenesis. This tumor suppressive action is achieved by the induction of cellular senescence. We found that Myc expression results in elevated levels of PML and PML-nuclear bodies, and increased expression of senescent markers, including p21, p16 and H3K9me3. Importantly, E6AP expression is elevated in >60% of human Burkitt lymphomas, and down-regulation of E6AP in B lymphoma cells restores PML expression with a concomitant induction of cellular senescence. Our findings demonstrate that E6AP regulation of PML-induced senescence is essential for B-cell lymphoma progression. This provides a molecular explanation for the down-regulation of PML in Non-Hodgkin lymphomas, thereby suggesting a novel therapeutic approach for restoration of tumor suppression in B-cell lymphoma.

Regulation of STEAP1 Expression in Prostate by Sex Steroid Hormones

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Introduction: Six transmembrane epithelial antigen of the prostate 1 (STEAP1) is an overexpressed gene in prostate cancer. It is localized in the plasma membrane of epithelial cells, specifically in cell-cell junctions. There are evidences that STEAP1 may be a useful marker for several types of cancer with different doses of DHT or E2 for several periods of time. The possible signaling pathways were accessed by exposing LNCaP cells to flutamide, ICI and cycloheximide. In vivo, 3 months old wistar rats were divided into 4 distinct groups that included orchidectomised animals treated with either DHT or vehicle, and intact animals treated with E2 or vehicle alone. STEAP1 expression in each experimental group was carried out by Real-time PCR and Western blot. Immunohistochemistry was performed in tissue microarrays (TMAs) of human prostate cancer and non-neoplastic lesions. Correlation between STEAP1 immunoreactivity and clinical data was established using a SPSS program.

Results and Discussion: STEAP1 expression is inhibited by the presence of DHT and E2 for several periods of time. The possible signaling pathways were accessed by exposing LNCaP cells to flutamide, ICI and cycloheximide. In vivo, 3 months old wistar rats were divided into 4 distinct groups that included orchidectomised animals treated with either DHT or vehicle, and intact animals treated with E2 or vehicle alone. STEAP1 expression in each experimental group was carried out by Real-time PCR and Western blot. Immunohistochemistry was performed in tissue microarrays (TMAs) of human prostate cancer and non-neoplastic lesions. Correlation between STEAP1 immunoreactivity and clinical data was established using a SPSS program.

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Conclusion: STEAP1 is down-regulated by both E2 and DHT in LNCaP cells and in rat prostate, suggesting that STEAP1 may influence prostate cancer progression in an androgen- and estrogen-dependent manner.

Molecular Role of EGFR-mediated Docetaxel Resistance in Human Androgen-Independent Prostate Cancer

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Purpose: Almost all patients ultimately die of androgen-independent prostate cancer (APIPC). Within 3 clinical years, it was important to identify molecular alterations responsible for docetaxel resistance in prostate cancer (PC). Previous studies suggested multiple roles for epidermal growth factor receptor (EGFR) in the developing prostate, the mature prostate, and in androgen-responsive and androgen-independent malignant prostate cancer. However, the EGFR-mediated chemoresistant mechanism in human PC was not well-known. In the study, we explored the molecular characterization of EGFR-mediated docetaxel resistance in PC.

Materials and Methods: We have established a series of stable docetaxel-resistant sublines, PC/DX by chronically exposing PC3 to progressively increased concentrations of docetaxel. The docetaxel IC50 was determined by the MT assay and the median-effect equation. EGFR induced molecular events in PC/DX were examined by real-time PCR and Western blotting. Expressions of EGFR protein in human PC tissues were examined by immunohistochemistry. PC/DX tumor xenografts in athymic nude mice were treated Gefitinib for 3 weeks, and tumor volume was determined.

Results: The docetaxel IC50 of PC3 and PC/DX was 0.0098 and 1.33 mM, respectively, indicating that the docetaxel resistance of PC/DX was 8.5-fold of that of PC3. Cellular resistance to docetaxel was significantly associated with elevated EGFR and EGFR activation in PC/DX. The drug-resistance related markers of PC3/DX, including GSTM1, GSTT1, and EGFR, were upregulated in PC/DX than in PC3 cells, respectively. PC/DX with greater resistance to docetaxel had higher levels of EGFR in a dose-dependent manner. Expression of EGFR was highest in adeno PC/DX than in PC3. RWPE-1 and LNCaP cell lines. Similar results also were found in human PC tissues. Our data showed PC/DX cells reversed to docetaxel sensitivity by knockdown of EGFR expression. Contrarily, overexpression of EGFR or recombiant EGFR protein could rescue PC3 cells from the docetaxel-mediated cytotoxicity. We examined the effect of EGFR inhibitor on nude mice bearing established PC/DX xenografts. EGFR inhibitor (Gefitinib; 200 mg/kg once-daily, p.o.) showed antitumor activity in PC/DX xenografts, and revealed a significantly improved survival rate of nude mice as compared with PC/DX xenografts.

Conclusions: We indicate EGFR play an important chemoresistant role in docetaxel-resistant PC, and suggest that inhibiting EGFR activity can enhance therapeutic responses to docetaxel-based therapy.

Determination of Snail1 Paracrine Functions – Implication in Pro-tumorogenic Abilities on Colorectal Epithelial Cells Lines

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Background: Clinical and experimental data supports the idea that the active recruitment of stromal cells by tumor cells is essential for the generation of a microenvironment that actively fosters tumor growth. Snail1 is a zinc finger transcriptional factor that plays an important role in epithelial-mesenchymal transition (EMT)-mediated metastasis through the regulation of E-cadherin expression. E-Cadherin is downregulated in human colon epithelial cells when co-cultured with Snail1 expressing cells. Moreover, expression of Snail1 in the tumor stroma correlates with lower specific survival of cancer patients. The aim of this study was to determine the possible pro-migratory paracrine functions of Snail1 expressing cells.

Material and Methods: Primary Cancer-Associated Fibroblasts (CAFs) were isolated from T16 colon human cancer tumors. After CAFs establishment and growth, we analyzed mRNA Snail1 expressing levels by RT-PCR. Colon cancer cell lines SW480-ADH or LIM1215 were co-cultured with a panel of Snail1 expressing colon and fibroblasts cell lines, or primary CAFs, to study migration and proliferation changes. Co-culture analyses were carried out using trans-well culture system.

Results: All three of the Snail1 expressing cell lines tested induced higher cell migration and proliferation compared to Mock cells. However, the pro-migratory effect of Snail was higher when it was expressed in fibroblasts cells. Moreover, increase of proliferation index in SW480-ADH cells were observed when cells were co-cultured with Snail1 expressing cells. Again, the effect was higher, reaching a ten-fold increase, when Snail1 was expressed by fibroblasts, instead tumor colon cells. In addition, heterogeneous Snail1 expressing cell lines in SW480 were observed between different cell lineages. Co-culture of primary CAFs with LIM1215 cells induced a significant increase in LIM1215 migration. We could observe significant differences of fibroblasts-derve pro-migratory effects on colon cancer cells among CAFs from different patients. These data suggest a direct correlation between CAFs pro-migratory potential and mRNA Snail expression. This data suggests that CAFs pro-tumorogenic abilities on the colon tumor cells are determined, at least in part, by Snail1 expression.

Conclusions: Together, these experiments demonstrate the Snail1-dependent paracrine pro-migratory and proliferative effects of fibroblasts on colon cancer cells. Future analysis will include gene expression profile, proteomic analysis and secretor phenotype study of Snail1 expressing cells, as well as primary CAFs, trying to characterize the cross-talk between colorectal cancer cells and fibroblasts.

Breast Cancer Stem Cells Glycolytic Metabolism and Response to Chemotherapy

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Background: Breast cancer is stratified into tumors that express hormone receptors, HER-2 positive tumors, and, tumors that do not express receptors (TN). Cancer stem cells were identified as responsible for tumor initiation, progression, resistance to therapy and recurrence of disease. Culture of breast tumor cells under special conditions leads to formation of spherical colonies, called mammospheres, which have properties of stem cells that can be confirmed by in vivo tumorigenic capacity. This project aims to identify breast tumor cells with characteristics of stem cells, to understand their role in chemotherapy response, correlating with their molecular profile in what concerns gene expression.

Material and Methods: Cells lines MCF7 [ER*], HCC1954 [ER*, PR*, HER-2*] and HCC1806 [TN] were used. Mammosphere forming protocol was performed. Cell cultures were injected in Balb-c nu/nu mice to assess tumorigenic capacity. Surface markers expression such CD24, CD44 and CD133 was characterized by flow cytometry. Cells were subjected to several concentrations of carboplatin (Carb), doctalexin (Doc) and epirubicin (Epi), and evaluation of proliferation was performed by MIT or Alamar Blue assays. BF-FDG was used to evaluate glycolytic metabolism.

Results: Cell lines formed spheres in suspension and, when placed in adherent culture conditions, adopt similar morphological of the cell lines that originated them. Cells with characteristics of stem cells obtained are able to form tumors. After 72 hours of drug incubation was found that cells that express receptors [MCF7, IC50: 244 mM Carb; Doc: 1.15 mM; Epi 173 mM] are more sensitive to chemotherapy than cell with characteristics of stem cells derived from the latter [IC50: 257 mM Carb; 6.37 mM Doc; Epi 228 mM]. However, in cells with stem cell characteristics derived from TN, [IC50: 41.2 mM Carb; Doc <50 mM; Epi 140 mM] might be less resistant than TN cells [IC50: 59.13 mM Carb; Doc 3.65 mM; 190 mM Epi]. Cell viability preliminary results showed that cells that overexpress HER-2 activate apoptosis death pathway, being Doc the most lethal. However, in TN Epi is the most effective being activated both apoptosis and necrosis. Concerning glycolytic metabolism, cells with stem cells characteristics derived from TN have higher radiotracer uptake reaching the peak at 60 min (3.70±0.83%), than TN (peak: 25±0.10%, 60 minutes). The same relation was observed for cells with characteristics of stem cells derived from cell that express receptors (peak: 3.42±1.06%, 60 minutes).

Conclusions: Cells with stem cells properties derived from cells that express hormone receptors are less susceptible to chemotherapy, evidencing their importance in resistance. However in TN cells this trend is not observed. Glycolytic metabolism was more prevalent in cells with stem cell properties, regardless the expression of receptors.

Metformin in the Therapeutically Achievable Plasma Concentration, Decrease Malignant Cell Survival Mostly Additively to the PBMC or to Treat In vitro Antitumor Activity

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Background: Epidemiological studies indicate that the therapy of diabetes mellitus type 2 (T2D) with metformin is associated with a reduction in the cancer risk. The aim of this work was to determine: the antiproliferative action of maximally achievable plasma metformin concentrations (present in patient's
Role of HMGA1 Pseudogenes in Human Cancer

Role of Sam68 in Modulating the Tumorigenic and Metastatic Potential of Breast Cancer Initiating Cells

Material and Methods: To six patients with T2D or hyperglycemia, and nine healthy volunteers were included in the study. Metformin hydrochloride was produced by Harman Finchem. Human: HeLa, MDA-MB-453, Her-2 positive MDA-MB-361 cells, K562 cells, Jurkat cells, were used as the target cells. Nutrient medium was RPMI 1640 with 10% of fresh human plasma. Determination of cell survival was done by MTT test.

Results: Metformin (2 mM) in nutrient medium with 10% of human plasma, after 72h of continuous action, significantly decreased cell survival: which was (69.02±5.5)% for K562 cells, while for MDA-MB-453 cells it was (86.97±2.7)%). The enhancement of the antitumor action of PBMC to malignant MDA-MB-361 cells was observed in the presence of the plasma of patient one month after the therapy with metformin in relation to the data obtained in the presence of plasma of the same patient before the therapy. The survival of target MDA-MB-361 after continuous incubation with metformin, and PBMC (for 24h) was lower than after their incubation with PBMC only, metformin action was mostly additive and synergistic one to the PBMC action. Pretreatment (for only 24h) of human breast cancer MDA-MB-361 cells with various concentrations metformin on the subsequent trastuzumab antitumor action (for next 48h) on these cancer led to the very mild, but additional one, decrease in target cell survival.

Conclusion: Metformin in the therapeutically achievable plasma concentration could decrease malignant cell survival through direct antiproliferative action, mostly additively to the antitumor PBMC or to trastuzumab antitumor action.

Material and Method: We demonstrate that HMGA1P6 and HMGA1P7 overexpression led to the inhibition of cell proliferation and apoptosis. Therefore, we have focused our studies to investigate the relationship between the mRNAs produced by the HMGA1 oncogene and its HMGA1P pseudogenes (HMGA1Ps) and the critical consequences of these interactions.

Results and Discussion: We observe that HMGA1Ps are biologically active. These findings attribute a novel potential, interfering simultaneously with CICs growth, chemoresistance and metastasis, whose investigation will allow the identification of specific molecular targets for innovative selective cancer therapy.

Desmoglein 3 regulates Cancer Cell Migration Through PKC-Dependent-ezrin Activation

Desmoglein 3 (Dsg3), also known as the pemphigus vulgaris antigen (PVA), belongs to desmosomal cadherin subfamily and mediates cell adhesion in the desmosomes. Dsg3 expression is up-regulated in squamous cell carcinoma (SCC) and is a good discriminating SCC marker for clinical staging of cervical sentinel lymph nodes in head and neck SCC. However, its biological function in cancer metastasis remains largely unknown. In this study we describe a novel phosphorylation mechanism of ezrin-Thr567 induced by Dsg3 that is likely contributing to the accelerated cell invasion and metastasis in SCC.

Material and Method: A431 and SqCC/Y1 with overexpression or knockdown of Dsg3 were utilized. Cell migration and invasion was assessed by transwell and organotypic invasion assays. Cell morphology was visualised by confocal microscopy. Protein expression and interaction were analysed by Western blotting, co-immunoprecipitation, proximity ligation assay and FRET. Pharmacological inhibitors were used to determine the signal pathways involved.

Results and Discussion: We showed that increased expression of Dsg3 in cancer cell lines promotes cell migration and invasion with concomitant...
augmented membrane protrusions and cell spreading. Dsg3 colocalizes and forms a complex with ezrin (also phosphorylated form) at the plasma membrane that seemed to be required for proper interaction between ezrin and F-actin since Dsg3 silencing caused significant reduction of the colocalization between ezrin/F-actin or CD44 and collapse of membrane protrusions. Prevention of Dsg3 elicited an amplified phosphorylation of the threonine residues at the C-terminus of ERM proteins, particularly ezrin-Thr567. Furthermore, we showed that the enhanced ezrin phosphorylation by Dsg3 could be abrogated substantially by various pharmacological inhibitors including those for PKCs and ROCK that are known to activate ezrin.

Conclusion: Our data suggest a novel signaling role for Dsg3 that acts as a transmembrane protein in regulating ezrin activation through various signal molecules including PKCs that are essential in actin-based cell migration, cancer metastasis and progression.

188 Isoenzyme-specific PFK-2/FBPase-2 Inhibition as an Anti-cancer Strategy
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Background: The up-regulation of glycolysis, even under aerobic conditions (Warburg effect), is an almost universal feature of primary and metastatic cancers, correlating with poor prognosis and increased tumour aggressiveness. Phosphofructokinase-1 (PFK-1) catalyses the first irreversible step of glycolysis, namely the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. PFK-1 is activated by fructose-2,6-bisphosphate, a product of the kinase activity of four isoenzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. PFK-1 (PFKFB1-4). Targeting these isoenzymes may provide opportunity for the development of antitumour agents, such as ROCK that is known to activate ezrin.

Materials and Methods: The effects of different concentrations of cigarette smoke and benzo(a)pyrene at different time interval were assessed on two lineages of colon cancer cells (HCT-15 and HT-29). The effect of cigarette smoke and benzo(a)pyrene on cell viability, reactive oxygen species, cell membrane integrity, cell apoptosis and proliferation was assessed by MTT, DCFHDA, DHE, DHR123, FDA, Annexin V-PI, and CYQUANT kit respectively. We screened both cell lines for different PFLA2 isoforms expression by using RT-PCR.

Results: The cell viability, cell membrane integrity and proliferation, deteriorated with time and increase in cigarette smoke and benzo(a)pyrene concentration which may be due to increased ROS production and apoptosis. Both the cell lines expressed all the PFLA2 isoforms except IIA, IIE, V, and VII. Expression of IB and IVA were increased with higher concentration of benzo(a)pyrene in HT-29 and HCT-15 cells respectively whereas increase in IBD were cell specific.

Conclusion: It seems that at least IBD form of PFLA2 and ROS is responsible for inflammatory reaction induced by cigarette smoke and benzo(a)pyrene in the colon cancer cells.

190 The Mucin MUC4-mediated Chemoresistance of Human Pancreatic Cancer Cells to Gemcitabine and FOLFIRINOX
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Introduction: Pancreatic cancer (PC) is one of the most deadly cancers in western countries. The fluorinated analog of deoxyctydine, gemcitabine (Gemzar®), is the main chemotherapy in PC, but survival remains weak mainly because of the high resistance of tumors to the drug, FOLFIRINOX, a more aggressive protocol combining 5-fluorouracil (5-FU) / irinotecan / oxaliplatin / leucovorin, gave recent promising results despite heavy side-effects. Deciphering the mechanisms responsible for PC cell resistance will help improve drug efficacy and allow us to propose more efficient therapies. Recent works have shown that the mucin MUC4 may confer an advantage to pancreatic cancer cells by modifying their susceptibility to chemotherapeutic drugs. However, the mechanisms underlying MUC4-mediated chemoresistance remain poorly understood.

Material and Methods: CAPAN-2 and CAPAN-1 adenosaccharomatous PC cell lines were used to establish stable MUC4-deficient clones (MUC4-KD) by retroviral interference. Measurement of the IC50 index to gemcitabine, 5-FU, oxaliplatin and irinotecan (SN-38) were performed using tetrazolium salt combined with evaluation of the apoptotic index (Annexin V) by flow cytometry and the Bax/Bcl-XL ratio by western-blotting. Expression of markers of gemcitabine metabolism: JCK, HENT1, HCNT1-3, RRM1/2 and MRPs was evaluated by quantitative RT-PCR. Activation of Erk1/2, JNK and NF-κB pathways was measured by western-blotting.

Results: MUC4 knocking-down in CAPAN-2 and CAPAN-1 PC cells led to an increase of their sensitivity to both gemcitabine and 5-FU compared to control Mock cells. This was correlated to an increase of the Bax/Bcl-2 ratio and apoptotic rate. No significant changes in sensitivity to oxaliplatin and irinotecan were observed. Upregulation of MRPs, HNT1 and HCNT1 and down-regulation of MRP4 expression was observed in MUC4-KD cells. Only HNT1 alteration was correlated to MUC4 expression and cell sensitivity to gemcitabine. Moreover, activation of MAPK, JNK and NF-κB pathways decreased in MUC4-KD cells in which NF-κB pathway was found to play an important role both in sensitivity to gemcitabine and in HNT1 regulation. Finally, in tissues from patients with pancreatic adenocarcinoma, MUC4 expression was significantly conversely correlated to that of HNT1.

Conclusions: Our work describes a new mechanism of pancreatic cancer cell resistance to gemcitabine in which the MUC4 mucin negatively regulates the HCNT1 transporter expression via the NF-κB pathway, making MUC4 and HNT1 as good potential targets to ameliorate the response of pancreatic tumors to gemcitabine treatment. Influence of MUC4 on 5-FU, oxaliplatin and irinotecan efficacy will have to be further investigated.
**192** EZH2 and GATA3 Play Opposing Roles in Controlling the Differentiation State of Basal-like Breast Cancer Cells

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**Background:** Breast cancers are phenotypically heterogeneous, exhibiting varying degrees of cellular differentiation and clinical outcomes. Tumors of the basal-like subtype are highly aggressive, poorly differentiated, and display a ‘bi-lineage’ progenitor-like phenotype. Despite their clinical importance, the pathways directing their unique progenitor-like differentiation remain poorly understood. We therefore hypothesized that EZH2 is involved in controlling the progenitor-like differentiation state of these tumors.

**Materials and Methods:** To explore our hypothesis we have used a panel of breast cancer cell lines derived from basal-like breast tumors, constituting a model for the disease. Using Lentivirus vectors we have over-expressed or silenced EZH2 in the model cell lines, we then characterized the cells by examining differentiation markers and expression signatures. Progenitor traits we evaluated using spheroid formation assay in matrigel, and by examining the expression of epithelial markers. To validate the role of EZH2 in vivo we have injected the treated cells into the fat-pads of NOD-SCID mice and followed tumor growth rate and differentiation state.

**Results:** We have found that silencing of EZH2 in basal-like cells leads to a decrease in the basal-like expression signature, accompanied by a decrease in the expression of markers characteristic of these tumors. Moreover, we observed a decrease in the progenitor-like differentiation state of these cells, expressed by the loss of progenitor markers CD133 and CD117, a decrease in 3D sphere formation ability and decreased tumor mass. Interestingly, the proportion of cells displaying a ‘bi-lineage’ phenotype was also reduced upon the perturbation of EZH2. We found that EZH2 acts to maintain the basal-like state by repression of the luminal regulator GATA3, which drives the population towards a ‘mono-lineage’, better differentiated state.

**Conclusions:** Our findings thus reveal a novel role for EZH2 in maintaining the progenitor-like state of basal tumors, and establish the interplay with GATA3 as a medianing mechanism.

**Reference(s)**


**193** MiR-187 is an Independent Prognostic Factor in Breast Cancer

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**Background:** MicroRNAs (miRNAs) involved in cancer have now become the focus of much attention as they represent a new class of biomarkers and potential drug targets. Here, we describe an integrated bioinformatics, functional analysis and translational pathology approach to identifying and validating novel miRNAs involved in breast cancer progression.

**Materials and Methods:** Given a database of miRNA binding site motifs and gene expression levels determined by transcriptomic profiling, correspondence analysis, between group analysis and co-inertia analysis can be combined to produce a ranked list of miRNAs associated with a specific gene signature and phenotype. We have applied this approach to examine gene expression data from 40 [1] and 69 [2] breast cancer patients and have identified miR-187 as the principle miRNA associated with disease progression in these cohorts. Ectopic expression of miR-187 was subsequently carried out in breast cancer cell lines and expression of the miRNA was evaluated in two cohorts of breast cancer patients by locked nucleic acid in situ hybridisation (LNA-ISH) on tissue microarrays.

**Results:** Ectopic expression of miR-187 in MCF7 cells revealed a more aggressive phenotype, with cells displaying an ability to grow in anchorage independent conditions coupled with an increase in migratory and invasive potential. A significant increase in the expression of MMP13 in cells overexpressing miR-187 was also noted. In a test cohort consisting of 111 breast cancer patients, high expression of miR-187 (3+) was borderline significantly associated with poor overall survival in multivariate cox regression analysis (HR 2.445, 95% CI 0.962–6.211, p = 0.06). However, in a further validation cohort of consecutive breast cancer cases (n = 470), a highly significant association was seen between high miR-187 and poor breast cancer-specific survival (BCSS) (HR 2.798, 95% CI 1.518–5.157, p < 0.001).

**Conclusion:** We hypothesise that miR-187 may play a role in the overall progression of breast cancer and potentially in the induction of a more aggressive and invasive phenotype. In this regard, miR-187 may function as not only a marker of poor prognosis but may serve as a novel drug target in breast cancer patients.

**Reference(s)**


**194** Mutation Sequencing and Atomic Force Microscopy of Fibroblasts Treated With 4-hydroxytamoxifen

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**Introduction:** Accumulation of mutations in key regulatory genes, such as the human TP53 gene, as a result of oxidative DNA damage may result in cancer. Exogenous and endogenous DNA adducts have been implicated, for example 3-(2-deoxy-β-D-erythro-pentofuranosyl) pyrimidine (3′-dG) [1]. One route of dG adduct formation is from the reaction of malondialdehyde (MDA) with deoxyguanosine. M1G adducts can be detected and quantified by measurement of free M1G (pyrimido[1,2-a]purin-10(3H)-one) in tissue extracts. Furthermore, M1G may be used as a biomarker for DNA damage and oxidative stress.

**Background:** M1G is a mutagenic and carcinogenic product of lipid peroxidation and as well as DNA adduct formation has been found to react with proteins to form protein adducts. The cytoskeleton is a major component of the cell and plays a major role in providing cells with structural and mechanical integrity. This research applied a multidisciplinary approach to investigate the toxic effects of M1G on human foetal lung fibroblast cells. In particular the work will focus on,
Overexpression of Integrin αvβ3 in Human Laryngeal Carcinoma (Hep2) and Tongue Squamous Carcinoma (Cal27) Cells: Adhesion, Migration, Invasion and Resistance to Antitumor Drugs

N. Stoegnhofer1, A. Brozovic2, D. Majhen1, C. Bressy2, K. Benihoud2, M. Herak S48 and the mechanical properties of the cells. The work will also investigate sequence variation in the TP53 gene and p53 expression levels. Finally, the work will investigate detection of free M-G by liquid chromatography–mass spectrometry (LC-MS) in vitro.

Materials and Methods: Following treatment of MRC5 and MRC5 SV2 cells with 0–100 μM MDA for 48 h, DNA was extracted and purified and codons 90–290 of the TP53 gene were amplified by PCR and sequenced. p53 expression in response to treatment with 0–200 μM MDA for 24 and 48 h was assessed by reverse transcription real-time PCR (RT-qPCR). The actin cytoskeleton of cells treated with 0–100 μM MDA for 48 h was labelled with fluorescent anti-actin antibodies and imaged using a Zeiss LSM510 confocal microscope. Indentation measurements of cells were made using an Aramis Research MFP-3D AFM to assess cell surface variation.

Results and Discussion: Sequence variation of codons 90–290 of the TP53 gene extracted from cells treated with MDA was assessed and mutational hotspots identified by comparison with the IARC TP53 database. TP53 expression levels were assessed by RT-qPCR in response to treatment with MDA. The results of both will be presented at the conference. Additionally, confocal microscopy and AFM images, and force-indentation measurements of cells subject to MDA treatment will also be presented. Further work is ongoing to improve the sensitivity of an LC-MS based method to measure the number of adducts formed under the same conditions.

Conclusions: The measurement of adducts is useful when looking at the initial effects on DNA. However, it doesn’t provide information on the long term consequences for the cell. In this study, the use of mutation sequencing and AFM has identified the effects of MDA at both the DNA and cellular level.

SIRT2 Functions as a Tumour Promoter in Osteogen Receptor Negative Breast Cancer

L.M. McGlynn1, J. Curle1, Z. Mohammadi1, S. Zino1, J. Edwards1, P.G. Shields1

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Introduction: The Sirtuins (SIRT1–7) are involved in DNA repair, cell cycle regulation and ageing. It is hypothesised that SIRTs may be involved in a number of age related diseases; in this case this will be aimed to determine the role of SIRT1–7 proteins as prognostic/predictive markers in oestrogen receptor (ER) negative breast cancer patients.

Methods: 153 ER-re breast tumours were analysed for SIRT1–7 tumour expression using an immunohistochemical approach. Prospective follow-up data was available. Two observers independently scored the staining using a weighted histoscore method. Statistical analysis was carried out using SPSSv18. For analysis patients were split into two groups those that expressed high and low levels of the protein of interest. High levels were defined as scores ≥ median value, whilst low levels were defined as scores < median value.

Results: Increased tumour size was associated with high expression of nuclear SIRT5 (p = 0.01) and low expression of cytoplasmic SIRT3 (p = 0.05). Increased tumour grade was associated with low expression of cytoplasmic SIRT3 and TST6 (p = 0.05 & p = 0.031) and nuclear SIRT4 (p = 0.039). Survival analysis revealed that only nuclear SIRT2 was associated with patient outcome. Patients whose tumours expressed high levels of nuclear SIRT2 had shorter disease free survival and overall survival, than patients whose tumours expressed low levels (p = 0.001 & p = 0.024). Multivariate analysis revealed that nuclear SIRT2 was independent of tumour grade, size and PR status in influencing disease-free and overall survival.

This study supports the differing functions of SIRT1–7 in cancer, as more aggressive tumours were associated with both low and high expression levels of the individual SIRTs, suggesting some act as tumour suppressors whilst others may serve as tumour promoters. The latter is pertinent to SIRT2 as high levels of this protein were associated with poor outcome. SIRT2 shuttles between the cytoplasm and nucleus, whilst in the nucleus it controls the cell cycle by acting as a mitotic check point. High levels of SIRT2 may indicate dysregulation of the cell cycle and perhaps accumulation of DNA damage. Over expression of SIRT2 can result in an increase in the number of multinucleated cells which could result in chemotherapy resistance and poor outcome.

Conclusion: SIRT2 regulates mitosis, overexpression is associated with poor outcome in ER-re breast cancer patients, and hence it may represent a novel therapeutic target for this subclass of disease.

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Cytotoxic Activity of a Cyclopamine Glucuronide Prodrug Against Glialoblastoma Cells

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Background: Glialoblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults. Median survival of patients is 15 months and effective therapies are needed. Abnormal activation of the developmental Hedgehog (Hh) pathway is observed in GBM and other cancers. Activation of this pathway component Smoothened (SMO) is associated with poor survival. The development of pharmacological modulators of SMO is hindered due to the high risk of drug resistance.

Methods: SMO expression was observed in C6 cells by immunofluorescence. Viability, clonogenicity and apoptosis tests indicated that the prodrug 2b in the presence of β-glucuronidase was toxic for C6 cells and C6-CSIC. This toxicity was specific to the Hh pathway, since GLI1 gene expression was downregulated by the prodrug 2b in the presence of β-glucuronidase in C6 cells and C6-CSIC. None of these effects was observed with the prodrug 2b in the absence of β-glucuronidase. Finally, the prodrug even in the presence of β-glucuronidase did not alter the viability of normal rat astrocytes.

Results: The cyclopamine glucuronide prodrug 2b is converted to an active drug by β-glucuronidase, an enzyme found in the necrotic area of GBM. This active cyclopamine is toxic to glialoblastoma cells and glioblastoma stem cells by targeting the Hh pathway, but does not impair viability of normal astrocytes. The prodrug 2b is a good candidate for in vivo investigations using C6 cells that generate tumors presenting a necrotic area when injected into the rat brain.

Breast Cancer-associated Fibroblasts Activated State is Regulated by Yes-associated Protein Activation Induced by Matrix Stiffness and Cytoskeletal Contractility

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The invasion of breast cancer cells into surrounding tissue is linked to its ability to metastasize and represents a major clinical problem. In addition to the cancer cells themselves, many other cell types influence this process; including endothelial cells, macrophages, and fibroblasts. Our study focuses on the role of stromal fibroblasts in promoting breast cancer spread. Contrary to normal fibroblasts (NFs), cancer-associated fibroblasts (CAFs) present a stable altered state that is documented to contribute to tumour progression. However, the molecular mechanisms governing the emergence and maintenance of the CAF phenotype remain poorly defined. We aim to analyse the role of CAFs in tumour progression in order to find key mechanisms that could prevent, and hopefully revert, their tumour promoting abilities.

We have isolated fibroblasts from normal, hyperplastic, adenomai, and adenocarcinous breast tissues and functionally characterized them. We have observed that when compared to NFs, CAFs present a higher steeper cytoskeletal contractility and enhanced three-dimensional matrix remodeling that promotes organotypic cancer cell invasion. In vivo, CAFs regulate angiogenesis, the immune response and cancer cell proliferation and invasion. Analysis of the transcriptome of this set of fibroblasts has revealed that CAFs are remarkably enriched in Yes-associated protein-1 (YAP1) target genes. Further analyses indicate that CAFs present a constitutively activation of YAP1 and that this persistent activation is linked to cytoskeletal contractility and matrix stiffness-sensing machineries. Moreover, YAP1 is essential for the characteristic activated state of CAFs and their key role in tumour progression. In agreement, activation of YAP1 is observed in the stromal fibroblasts of cancerous tissues of breast cancer in origin, and is highly correlated to poor outcome. These findings reveal a novel key mechanism governing the active role of CAFs in tumour progression and may present new molecular targets for intervention and diagnosis.

Identification of Cellular Binding Proteins for the EphB4 Receptor in Prostate Cancer Cells Using the Novel Proteomic Method 2D-NACE

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Background: Prostate cancer is the most prevalent cancer in males in the developed world. The development of new options for treatment is urgently needed. Important to achieving this is identifying new targets for novel therapies. The receptor tyrosine kinase EphB4 is significantly over-expressed in epithelial cancer cells, including in ~60% of prostate tumours. It has been shown to contribute to tumour development by promoting angiogenesis, cancer cell survival, invasion and migration in vitro and in vivo. Little is known about how EphB4 contributes to prostate cancer progression and the signalling pathways activated by EphB4 are potential key targets for the development of novel anti-prostate cancer strategies. As such, identification of proteins that specifically interact with EphB4 in prostate cancer cells will provide crucial insight into the function and mechanisms of activation of EphB4 and therefore, provide new approaches for blocking EphB4 action.

Material and Method: Our laboratory has developed and validated a new technique for isolating and identifying interacting protein partners. This technique, called 2-dimensional Native Affinity Compromised Electrophoresis (2D-NACE), is a sensitive and efficient method that allows the concurrent but separate isolation of individual native protein complexes that contain EphB4. This is achieved by separating protein complexes in two dimensions on native gradient polyacrylamide gels. The second dimension stacking gel contains an EphB4 specific antibody which interacts with EphB4 containing complexes and changes the mobility of these complexes. These complexes are then visualized by silver staining, excised, digested and the peptides identified by LC-MS/MS.

Results: Using this technique we have already identified in several EphB4-interacting proteins that have various roles in cancer progression such as cell proliferation, apoptosis and migration. EphB4 has also been implicated in many of these cellular processes suggesting these interactions are true positives. Validation studies to confirm these interactions are underway using co-localisation by confocal microscopy and immunoprecipitation pull-down assays.

Conclusions: 2D-NACE is a novel tool that can be used to identify native interacting protein complexes. These interacting proteins will offer new insights into the mechanisms of action of EphB4 in prostate cancer and identify opportunities for the development of targeted anti-cancer therapies.

Investigation of Cell Death in Caco-2 Colon Cancer Cells After mTOR Inhibition by Rapamycin

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Background: mTOR (mammalian target of rapamycin) is a serine-threonine kinase that regulates protein synthesis, cellular growth, differentiation and survival. In addition it play an important role in cell skeletal and ribosome reorganisation, meiosis, translation, cell cycle, apoptotic and autophagic cell death. Recent studies indicate the role of mTOR in tumorigenesis. It is found that mTOR induces cell growth and proliferation in cancer cells. The role of mTOR in colon cancer is still remain to be explained. Rapamycin is a macrolide antibiotic which blocks mTOR and is trialed in cancers included colon cancers. The aim of this study was to investigate the cell death (especially apoptosis and autophagy) in colon adenocarcinoma cell line (Caco-2) after inhibition mTOR by Rapamycin.

Material and Methods: Two groups were designed for this study: a. Group I: Control group (Caco-2), b. Group II: Rapamycin (10nm) incubated group. Immunohistochemical analysis was performed with antibodies for apoptosis (TUNEL/Annexin V and caspase-3, caspase-8, caspase-9) and for autophagy (mTOR, beclin-1, ATG-12).

Results: The antibody dyes were positive in the cytoplasm in both group. TUNEL/Annexin V and p33 was more intense and caspase-3 was moderate in group II and mTOR was reduced in the Rapamycin group.

Conclusion: It is very important to define the cell death type which have an pivotal role in cancer therapy. We suggest that the internal way of apoptosis is more efficient in Rapamycin treated colon adenocarcinoma cells. Future studies should aim at mTOR targeted therapies in colon cancers in order to improve the outcome.

Molecular Mechanisms of Bicalutin-induced Autophagic Cell Death in Human Colon Cancer Cells

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Background: Autophagy is a protein degradation system of the cells’ own lysosomes, and has attracted the interest of researchers in the field of cancer research because it induces programmed cell death. However, roles in autophagy in cancer are not yet fully determined. Accumulating evidence demonstrates that many natural products have potent anti-tumor effects on...
various human cancers. Baicalein was isolated from the traditional Chinese herb medicine, the roots of Scutellaria baicalensis Geolgi which was used against allergy, infection, hypertension, and cancer. In this study, we examined the anti-cancer efficacy and molecular mechanisms of baicalein in human colon cancer cells. We found that baicalein induced autophagy rather than apoptosis in colon cancer cells.

Materials and Methods: Human colon cancer cell lines, HCT-116 with wild type p53 and HT29 with mutated p53, were used to analyze the anti-proliferative effects of baicalein. The effects of baicalein on the proliferation of the cells were evaluated by WST-8 assay. Induction of apoptosis was analyzed by the cleavage of caspase 3 and PARP by western blot. Induction of autophagy was analyzed by western blot and immunocytochemistry using antibody against LC3. The expressions of autophagy-associated proteins were analyzed by western blot.

Results: The proliferation of HCT-116 cells was markedly inhibited by treatment with baicalein. In contrast, the same dose of baicalein did not affect the proliferation of HT29 cells. Cleavage of caspase 3 and PARP in both HCT-116 and HT29 cells that were treated with baicalein was not obvious. However, accumulation of LC3-II was found in HCT-116 but not in HT29 after baicalein treatment. We also found that the formation of autophagic vacuoles in the cytoplasm of HCT-116 cells but not in that of HT29 cells after baicalein treatment. To elucidate the molecular mechanisms by which autophagy was induced in HCT-116 but not in HT29, we analyzed the expression of damage-regulated autophagy modulator (DRAM) that is a p53 target gene encoding a lysosomal protein that induces autophagy. We found that DRAM mRNA expression was induced by baicalein in HCT-116 but not in HT29 cells. These results suggest that baicalein might induce autophagic cell death in p53-dependent manner in human colon cancer cells. We will perform the experiment to see if the knock-down of DRAM suppresses induction of autophagy in HCT-116 cells.

Conclusion: This study shows that baicalein has high anti-cancer efficacy by inducing p53-dependent autophagy and warrant further investigation toward possible clinical application in patients with colon cancer with wild type p53. This study also shows that baicalein induces cell death in a DRAM-dependent manner in human colon cancer cells. The mechanisms of deterioration are principally related to the generation of Reactive Oxygen Species (ROS), as well as the processes linked to the telomeres and by the processes of cellular death.

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Background: The index of respiratory competence (IRC) is the percentage of oxidative organisms in relation to the total organisms grown (oxidative and fermentative). One type of mitochondrial mutants in Saccharomyces cerevisiae is the so-called white petites, in which mitochondria are not functional. These cells can not get their energy through respiration (aerobic), so it must necessarily rely on the fermentation metabolism (anaerobic conditions).

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Immunohistochemical and Molecular Investigation of Stem-cell Markers – CD133 and C-Kit – in Pleomorphic Adenoma of Salivary Glands
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Background: Salivary gland neoplasms are originated from the various salivary gland compartments; in general they are histologically related to other aspects of salivary gland neoplastic lesions. Pleomorphic adenoma is a common biphasic benign salivary gland neoplasm composed by epithelial and myoepithelial cells and a complex stroma. Its varied structural and architectural aspects suggests the participation of stem cells in the tumour composition. Additionally, pleomorphic adenoma originates from intercalated duct of salivary glands, a region reputed to host the regenerative/ stem-cell compartment of these glands. The present work investigated stem-cell markers (CD133 and C-kit) in pleomorphic adenoma and in specific cases developing human salivary glands using immunohistochemistry and real-time RT-PCR.

Material and Methods: One hundred and one cases of pleomorphic adenomas were included in the study – 55 cases were paraffin embedded and 66 specimens were fresh frozen tissue. From the total of 101 cases, 14 were paired (paraffin embedded and frozen tissue). A pool of normal salivary glands was used as controls. Salivary gland specimens dissected from 20 human fetuses were also used.

Results: All cases of pleomorphic adenomas were positive for the stem-cell markers studied. Neoplastic luminal structures were positive for CD133 and C-Kit. In focal areas, isolated cells were positive for CD133 and C-Kit. In foetal salivary glands, these markers were almost all restricted to the intercalated duct region. Increased expression of C-kit stem-cell markers was observed in pleomorphic adenoma specimens using real-time RT-PCR technique when compared with normal salivary gland controls. A specific expression pattern was not observed for CD133, when compared with normal salivary gland tissue – in some cases of pleomorphic adenoma there was an increased expression of both factors, whilst in other cases their expression was decreased or not altered. These patterns were not associated with tumor behavior or recurrence.

Conclusion: Pleomorphic adenoma cells share similar markers with stem-cells, although it is possible to confirm that neoplastic cells bear the same characteristics of multipotency.

Phospholipid Scramblase 1 Sensitizes Mantle Cell Lymphoma to Apoptosis Induced by Several Drugs
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Introduction: Mantle cell lymphoma (MCL) is usually an aggressive lymphoid malignancy representing 5–10% of all non-Hodgkin’s lymphomas, with an expected overall survival still in the range of only 4–5 years. Therefore, it is necessary to develop new therapeutic strategies and identify new markers of susceptibility/resistance of potential clinical value to improve the outcome of MCL patients. The purpose of this study is to evaluate the potential role of phospholipid scramblase 1 (PLSCR1) as a new marker of susceptibility to drug-dependent apoptosis in MCL. PLSCR1 is a protein that promotes the pro-apoptotic bimolecular membrane exposure of phosphatidylserine and our preliminary data indicate that PLSCR1 is strongly up-regulated in MCL cells undergoing apoptosis following treatment with 9-cis-retinoic acid (RA)+interferon-alpha (IFN-α).

Material and Methods: Mino, SP-53 and Jeko-1 MCL cell lines and primary MCL cells were used. Tumor biopies were analyzed for PLSCR1 expression by immunohistochemistry. RT-PCR experiments allowed the analysis of transcriptional modulation of PLSCR1. Proteins expression and STAT 1, MAPK and Akt pathways involvement was assessed by immunoblotting analysis. We took advantage from multispectral imaging flow cytometry to study CD20 expression and phospho-STAT1 nuclear translacion, and to evaluate apoptosis. We studied PLSCR1 involvement in mediating drugs pro-apoptotic effects was studied using MCL cell line in which PLSCR1 silencing was obtained by infection with pSUPER.retro.neo+GFP vector containing a specific shRNA

Results: Immunohistochemical analysis of 28 tumor biopies and 4 primary cultures revealed a variable expression of endogenous PLSCR1 in MCL. In particular, 9 samples showed more than 10% of neoplastic cells expressing this protein. We demonstrated that the expression of PLSCR1 is inducible in primary MCL cells by RA/IFN-α combination in association with the induction of apoptosis. The treatment induces PLSCR1 up-regulation, at both transcriptional and protein levels, also in MCL cell lines. We demonstrated the involvement of STAT1 as transcriptional mediator and its dependence on MAPK and Akt pathways activation. Notably, the exposure of RA/IFN-α preconditioned MCL cells to DOxorubicin (DOXO) or Bortezomib (BTZ) induced a further increase in PLSCR1 expression, which was associated with markedly enhanced drug-dependent apoptosis. Moreover, experiments carried out with cell lines in which PLSCR1 was silenced demonstrated that RA/IFN-α−conditioned MCL cells to DOXO and BTZ treatment, but not to Rituximab, as a consequence of RA/IFN-α-dependent CD20 down-regulation.

Conclusions: These results highlight the role of PLSCR1 in mediating drug-dependent MCL cell apoptosis and in cell sensitization to anti-tumor agents currently used in the clinical practice for the management of MCL, and stimulate further studies to identify clinicopathological correlations with endogenous PLSCR1 expression in MCL.

The BRAF-MEK Pathway Perturbs the Expression of ZEB Proteins in Malignant Melanoma
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Introduction: During an epithelial-mesenchymal transition (EMT), epithelial cells lose epithelial features and acquire mesenchymal characteristics. Two important transcriptional regulators of EMT include ZEB1 and ZEB2. A hallmark of EMT is the loss of cell cohesion, allowing cells to become motile, linking this process to tumour metastasis. In malignant melanoma, EMT redirects tumour cells from contacts with epidermal keratinocytes to interactions with dermal cells. This promotes transition from a horizontal to a vertical growth phase.

Material and Methods: Immunohistochemical analysis of human nevi, primary melanomas and matched primary and metastatic melanomas was performed. The MEK inhibitor, UO126, was used to inhibit the BRAF-MEK pathway. Protein expression was examined following application of inhibitors or siRNA knockdown. DNA demethylation was induced with 5-aza-2′-deoxycytidine. Results and Discussion: The loss of ZEB2 and acquisition of ZEB1 expression was identified during melanoma progression. The expression of ZEB1 and ZEB2 was found to be controlled by the BRAF-MEK pathway, with the transcription of ZEB2 being strongly repressed by activated MEK. In turn, ZEB2 was found to repress ZEB1. The only functional transcriptional repressor of E-cadherin was identified as ZEB1. Additionally, genomic DNA demethylation was shown to cooperate with ZEB1 depletion to enhance repression of E-cadherin.

Conclusion: These findings describe a novel pathway linking melanoma-initiating mutations in BRAF or NRAS oncogenes to EMT.

The Involvement of DNA Double-strand Break Repair in Receptor Tyrosine Kinase RON-mediated Cancer Cell Response to Hypoxia
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Background: Receptor tyrosine kinases (RTK) are one of major proto-oncogene classes and play a crucial role in many cell regulatory processes. Receptor of Origin Nantaise (RON) is a member of c-Met RTK family, and has been proved to play an important role in the pathogenesis of human cancer. We recently reported that RON can dimerize with EGFR and translocate to the nucleus as a transcriptional regulator when cancer cells are exposed to serum starvation. This study was designed to examine the molecular mechanisms of RON in the protection of cancer cells from hypoxia injury.

Material and Methods: TSGH8301 bladder cancer cell line was analyzed for their growth, subcellular localization of RON, and interaction with HIF-1α in response to hypoxia. Then, nuclear extract of hypoxic cells was co-immunoprecipitated with RON antibody and analyzed by high-performance liquid chromatography/mass spectrometry/mass spectrometry (co-IP-HPLC/MS/MS).

Results: We demonstrated a time-dependent nuclear translocation of RON as early as 1 hr after hypoxia, and greater than 50% of RON signals were accumulated in the nuclei within 24 hr. Nuclear RON associates with HIF-1α at 3 hr after hypoxia. The co-IP-HPLC/MS/MS identified a total of twenty-eight candidate interacting proteins. Among them, we were interested in ATP-dependent DNA helicase 2 (Ku70) and DNA-dependent protein kinase (DNA-PK) that were immunofluorescence staining and western blotting of subcellular fractions demonstrated a time-dependent increase of g-H2AX in TSGH8301 cells after hypoxia. There was a time-dependent increase of phospho-ATM as well.
Conclusions: RON receptor protein can be translocated into nuclei of cancer cells in response to hypoxia and interact with HIF-1α. Activation of non-homologous end joining repair system in response to hypoxia. The observation provides novel therapeutic targets for cancer cells with over-expression of RON.

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[215] Gene Expression Analysis by QPCR: Experimental Determination of PCR Detection Limits

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Background: Cytochromes P450 (CYP) are responsible for activation of pro-carcinogens into DNA damaging compounds that can induce mutation and tumor development. Site specific expression of CYPs can contribute to tissue susceptibility to environmental carcinogens. CYP expression can be analyzed in small amounts of tissue through qPCR. qPCR is the gold standard method to assess gene expression, if it is expressed above the detection limit. Although the detection limit of this technique was theorized to be three copies of template, there is no experimental proof of this. Here we experimentally demonstrate this detection restriction.

Materials and Methods: Tissues from three sites of five eight week old Wistar rats (liver, lower gingiva, hard palate) were collected and immersed in TRIzol. Total RNA was extracted and purified, using RNeasy kit. Increasing RNA amounts (from 10ng to 2560ng) were used in RT-PCR reactions. Primer sequences for β-actin, CYP2A3 and CYP2E1 produced amplions with ideal length (80–150bp). These were cloned on PGMT vector. Plasmids were extracted and inserted were sequenced, linearized and used to construct controls for absolute quantification. qPCR was performed with Rotor-Gene SYBR Green PCR kit (Qiagen, Germany).

Results: Data series organized according to detection probability (number of detections/number of reactions made), revealed that the group with detection probability different from 1 (n = 33) showed template inputs not different from the theoretical lower limit of three copies per reaction (2.89 copies, Wilcoxon Signed Rank Test: p = 0.9). So, detection probability appears to indicate that a particular gene is at the theoretical lower limit of detection by PCR in our qPCR system. The other group, where detection probability equals 1 (n = 48), was represented by the median value of 1365 copies. This group showed template inputs statistically different from three copies per reaction (Wilcoxon Signed Rank Test - p < 0.0001) and statistically higher template inputs (Mann-Whitney U test).

Conclusion: We showed that the previously theoretical devised limit is real, however used to describe a class of qPCR reactions with detection probability of less than 1, in the unit, where stochastic events and effects of this order are present due to very low template input at the beginning of these reactions. The present methodology can be used to assess the quantification limits in other qPCR platforms.

[214] Ouabain Inhibits in Vitro Proliferation of Tumour-associated Fibroblasts and Breast Cancer Cell Lines

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Background: It has become clear that progression of carcinomas depend not only on alterations on epithelial cells, but also on changes of microenvironment. Solid tumors survival and development is often based on vascular network sustaining such an intense metabolic process. Main purpose of our study was investigation of pro-angiogenic factors (VEGF) secreted within tumor environment and inhibition of autocrine and paracrine effects acting on both tumor cells and tumor associated fibroblasts (TAF).

Material and Methods: Bone marrow-derived MSCs, TAFs and tumor cell line SK-BR3. Level of VEGF expression was determined using ELISA method, while immunocytochemistry revealed positive staining in case of TAF and SK-BR3 cell line. Ouabain, Na+/K+ATPase inhibitor, was used in concentrations ranging from 10μM to 1mM and the amount of cellular utilization was determined by HPLC. MTB-based viability assay showed decreased proliferation rates for higher concentration of Ouabain. Flowcytometric analysis investigated expression of phenotypical markers, including CD106, CD44, CD29, CD117, Cx43 for MSCs and TAFs, and Her2, CD44, VEGF-R and CD29 for SK-BR3 cells. Gene expression of v1 and j1 subunits of Na+/K+ pump, as well as VEGF were determined in genuine cells and treated ones using qRT-PCR method. Immunocytochemistry investigated presence of v1 and j1 subunits of Na+/K+ pump, VEGF and adhesion molecules.

Results: Secretion of VEGF was significantly reduced in Ouabain-treated SK-BR3 and TAFs. Although level of expression for v1 catalytic subunit was increased in SK-BR3, we could not find presence of corresponding protein, reaffirming also in high level of Ouabain detected expression of subunits. Expression of adhesion molecules was decreased, and we found profound changes in phenotypic profile of both TAFs and SK-BR3 cells.

Conclusion: Based on their anti-angiogenic and anti-proliferative activity, Na+/K+ pump inhibitors could open novel anti-tumoral therapeutic strategies.

[213] The Role of MRNA-196a and HOXB9 in Head and Neck Cancer

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Introduction: HOX genes mediate organogenesis and determine developmental patterning along the antero-posterior axis. A number of conserved mRNA families, such as miR-10 and miR-196, are present within the HOX gene clusters. Recently it has been observed that the miR-196 family directly targets a number of the HOX genes and other targets like KRT5 and ANXA1, which may play important roles in oncogenesis. HOXB9 is expressed early in embryogenesis and is involved in forelimb development. Over-expression of HOXB9 in breast cancer increased metastasis, and in some cancers this is under the control of the Wnt/Tcf pathway. Preliminary microarray data suggested over-expression of a number of HOX genes (including HOXB9) and miR-196a in Head and Neck Squamous Cell Carcinoma (HNSCC). HOXB9 and miR-196a-1 are spatially closely related on 17q21.

Methods: Quantitative-PCR (qPCR) of miR-196a and all 4 HOX gene clusters was performed in the normal (NOK), oral pre-malignant (OPM), HNSCC cell lines. miR-196a expression was measured by qPCR in laser-captured FFPE tissue samples. HOXB9 expression was assessed by western blot (WB) in NOK, OPM and HNSCC cell lines and in tissue samples. Functional assays for proliferation, adhesion, migration and invasion were conducted after transfection of anti-miR-196a into NOKs, miR-196a-1 and HOXB9 siRNA into OPM and HNSCC cells and pre-miR-196a into NOKs.

Results: miR-196a was up-regulated 600-4000 fold in OPM and HNSCC cells compared with NOKs. FFPE HNSCC tissue samples showed 3x up-regulation in miR-196a when compared to normal (p < 0.05). Transfection of anti-miR-196a resulted in no change in proliferation of HNSCC cells but reduced migration, invasion and adhesion (p < 0.001). Immortalised NOKs transfected with anti-miR-196a showed no significant change in proliferation, adhesion or invasion but migration delivery was increased (p < 0.01). The expression of the HOX genes, qPCR and WB showed that HOXB9 was highly up-regulated. HOXB9 siRNA reduced the proliferation, migration and invasion (p < 0.01) but had no significant effect on the expression of fibrostenin in OPM cells. Initial iHC data showed increased HOXB9 expression in HNSCC tissue compared with normal tissue.

Conclusions: Our data shows that miR-196a and HOXB9 are over-expressed in HNSCC; it also suggests that they may be co-regulated due to their close proximity. Over-expression of miR-196a promotes adhesion, migration and invasion in HNSCC cells whereas HOXB9 up-regulation promotes proliferation, migration and invasion in OPM cells.

[212] The Role of MDGI in Glioma Progression

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Background: Despite of advances in cancer therapies, the prognosis for high-grade glioma patients is poor due to the infiltrative growth of tumor cells. Therefore, in order to develop novel therapeutic strategies it is essential to clarify the molecular mechanisms underlying the invasive behavior of these tumors. With in vivo phase display technology we have identified a peptide, CooP, which, after systemic delivery, specifically homes to malignant brain tumor islets harboring co-opted blood vessels. Mammary-derived growth inhibitor (MDGI) was validated to be an interacting partner for our peptide. This protein is involved in fatty acid metabolism but its role in glioma development is currently unknown.

Materials and Methods: To facilitate functional studies of MDGI, stable human glioma cell line (U87MG) overexpressing MDGI is used in 2D and 3D cell culture systems as well as in mouse xenograft models. In the gene expression patterns in response to MDGI expression, both in vivo and in vitro conditions, are studied using gene arrays. In addition, MDGI’s effect on cell metabolism will be investigated further.
Results: Our preliminary results showed that MDGI is expressed in a grade-dependent manner in clinical human astrocytoma samples. Furthermore, our first in vivo experiments showed that MDGI overexpression increases cell invasion in fibrin matrix. The cells overexpressing MDGI also showed a higher tendency to form colonies in soft agar, which together with the increased invasiveness supports the role of MDGI in cancer progression. Interestingly, immunohistological analyses of the glioma xenografts overexpressing MDGI revealed increased amount of blood vessels. Also upregulation of certain metabolism-associated proteins were observed in the MDGI overexpressing glioma xenografts, which might indicate the importance of MDGI in cellular bioenergetics.

Conclusion: Our results using CooP homing peptide suggest MDGI to be a novel biomarker for malignant gliomas. In addition, since increased invasiveness is a known hallmark in cancer development, our results also demonstrate a functional role for MDGI in glioma progression. The potential effect of MDGI on cell metabolism might also benefit cancer cells in the challenging environment of tumor tissue.

[217] Essential Role of Diacylglycerol Kinase Activity in Breast Cancer Growth and Metabolism
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Introduction: Oncogenic mutations allow the metabolic reprogramming of tumor cells to acquire survival and proliferative advantages over normal cells. mTOR, a master regulator of cell energy and nutrient availability, lies at the core of the PI3K/AKT pathway, one of the most frequently mutated in cancer. As a result, mTOR controls key aspects of tumor metabolism including de novo lipogenesis.

Diacylglycerol (DG) and phosphatidic acid (PA) are two lipids that, beside their role as second messengers, constitute a central node in phospholipid and triacylglycerol synthesis. Thus, their levels need to be finely regulated. DG Kinases (DGK) catalyze the phosphorylation of DG into PA. In addition to their eminent metabolic role, DGK are also modulators of several signaling pathways whose deregulation can lead to tumor formation. However, their exact contribution to cancerous states and mTOR function has not been thoroughly determined.

Methods: To examine the effect of DGK on tumor growth and lipid metabolism, we used a panel of breast cancer-derived cell lines as a model in which to downmodulate DGK activity with a pharmacological inhibitor (RS50498) or by RNA interference (RNAi).

Results: In these cells, DGK activity is essential for preserving the phosphoinositide intermediates that fuel the PI3K/Akt/mTOR axis and contributes to the maintenance of the PI3K/AKT pathway, more sensitive to DGK inhibition. DGK activity was coupled to tyrosine kinase signaling and was elicited by several feedback loops, such as those reverted by rapamycin, an mTOR inhibitor. Finally, we found that interfering with DGK activity had a potent anti-proliferative effect both in vitro and in vivo.

Conclusions: Together our results indicate that DGK activity is indispensable for tumor growth and can constitute new strategies to target tumor lipid metabolism.

[218] Telomerase Inhibition by Constatinolide Decreases Alpha-fetoprotein Secretion by Hepatocellular Carcinoma C3a Cell Line – Possible Role of PI3K/mTOR/STAT3 Pathway
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Introduction: The alpha-fetoprotein (AFP) is expressed by the majority of patients with hepatocellular carcinomas (HCC). It serves to diagnose and maintain the balance between the use of glucose for energy generation and the Synthesis of Anti-oxidants.

Material and Method: We performed a siRNA-mediated screen to silence the glycolytic enzyme PFKFB4, an isoform of phosphofructokinase 2 (PFK2), to be selectively required for prostate cancer cell survival. PFK2 is a bifunctional enzyme that regulates the levels of fructose 2,6-bisphosphate, a strong allosteric activator showing any significant effect neither on AFP secretion nor at the mRNA level. The measurement of caspase-3, an apoptotic marker, showed no significant difference between control and treated C3a cells. In order to demonstrate the direct relationship of telomerase with AFP expression, the C3a cells were treated with 15 nM of hTERT siRNA that showed a 35% decrease of AFP secretion, in the mean. Interestingly, the inhibition of STAT3 by siRNA dramatically decreased the release of AFP in the supernatant while the inhibition of PI3K by wortmanin, GSK and PI828 and the inhibition of mTOR by the compound 401 showed a slight decrease of AFP secretion. An additional effect was also seen when coRAPY was added to the above mentioned inhibitors. Furthermore, the inhibition of protein kinase C, ERK, JNK and P38 MAP kinase did not show any significant effect.

Conclusion: The inhibition of telomerase and PI3K/mTOR/STAT3 signaling pathway decrease AFP secretion, possibly, by two independent mechanisms.
of the most important regulatory enzyme of glycolysis phosphofructokinase 1 (PFK1). We found that silencing of PFKFB4 increased fructose-2,6-
biphosphate levels selectively in the prostate cancer cells, suggesting that it was mainly functioning as a fructose-2,6-bisphosphatase. PFK2 isoforms can modulate the distribution of metabolites between glycolysis and the pentose phosphate pathway. Prostate cancer cells also showed lower NADPH and reduced glutathione levels after PFKFB4 silencing, resulting in enhanced oxidative stress and cell death.

Moreover, inducible depletion of PFKFB4 inhibited tumor growth in a xenograft model, indicating that it is required under physiological nutrient levels. Interestingly, PFKFB4 is expressed at higher levels in metastatic prostate cancer compared to primary site tumors in public data sets from human prostate cancer.

The requirement of PFKFB4 for cancer cell survival was not specific to prostate, since a subset of bone, brain, breast, colon, lung, ovarian and stomach cancer cell lines were also dependent on its expression for their survival. Conclusion: We found that the glycolytic enzyme PFKFB4 is essential for prostate cancer cell survival by maintaining the balance between the use of glucose for energy generation and the synthesis of anti-oxidants. PFKFB4 is also required to support tumor growth in vivo. Taken together, these results indicate that PFKFB4 is a potential target for the development of anti-neoplastic agents.

**[22] FER Kinase Promotes Breast Cancer Growth and Metastasis by Regulating Cell Adhesion and Migration**

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Introduction: The Feline Sarcoma (FES) and Fes-related kinase (FER) proteins are the two members of a unique family of non-receptor tyrosine kinases. FER has been shown to regulate cell cycle progression, cell adhesion and migration in multiple cell types, and has been implicated in prostate and hepatocellular cancer formation and metastasis. The aim of our study was to determine whether FER is involved in breast cancer development and progression.

Methods: We examined FER expression in 485 cases of invasive breast carcinoma by immunohistochemistry and used human breast cancer cells to determine the contribution of FER to cell adhesion, migration and invasion. Finally, a mouse xenograft breast cancer model was used to investigate the role of FER in tumor growth and metastasis.

Results: High FER expression was significantly correlated with tumor size, grade and mitotic activity. We found that basal and HER2-driven tumors have higher FER levels as compared to luminal carcinomas. High FER expression correlated with a significantly worse prognosis based on overall survival. Further, multivariate analysis revealed that high FER expression is an independent prognostic factor.

Conclusions: Our data indicate that FER is highly expressed in aggressive breast carcinomas and that high FER expression is an independent predictor of decreased patient survival. Moreover, we have shown that FER regulates tumour growth and metastasis in vivo.

I.A. Ivanova is a Research Fellow of the Terry Fox Foundation (Award #700041).


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Introduction: Melanoma is a highly metastatic tumor with early metastasis in distant organs like lymph nodes, lungs, liver and brain. In order to understand the complex process of metastasis and to identify molecules involved, suitable in vivo and in vitro models are essential. The aim of this study was to establish variants of the human melanoma cell line Mel-Juso with same genetic background and different metastatic potential.

Material and Methods: Mel-Juso cells were inoculated into the tail vein of athymic nude mice. Lung metastases were harvested, pooled, cultured in vitro and injected in another set of mice. Different melanoma variants were generated by repeated cycles of in vivo passage and migration. The obtained melanoma variants (L3 and L5) were characterized genetically and concerning the expression of a melanoma marker, certain Eph receptor tyrosine kinases, growth properties, and in vivo metastasis.

Results and Discussion: STR DNA genotyping showed no differences between the parental cell line and two selected metastatic variants. Moreover, no differences of the melanoma-associated chondroitin-sulfate proteoglycan and in Eph receptors could be detected. Interestingly, we detected a reduced proliferation in metastatic variants accompanied by reduced colony formation and adhesion to the extracellular matrix. These changes in metastatic properties despite of genetic similarity. Nevertheless, these in vitro differences between Mel-Juso and the metastatic variants could not be confirmed in an in vivo metastasis assay. Therefore, we started an additional cycle of in vivo passage with preparation of metastatic variants (L6) from individual lung metastases. By now 15 individual cell clones could be established derived from lungs of 4 individual mice and are currently analyzed concerning their cellular properties.

Conclusion: The generation of melanoma cell line variants with same genetic background and different metastatic potential showed no success when using pooled lung metastases. Further steps will focus on the generation of variants from individual metastases to better reflect the varying tumorigenic potential of individual melanoma cells. Using such variants would facilitate the identification of molecules involved in metastasis, which show promise to be potential targets for the diagnosis and therapy of melanoma.

**[223] Thrombospondin 2 Gene (THBS2) and Its Role in Ovarian Cancer**

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Introduction: THBS2 is multimodum Ca-binding extracellular glycoprotein that play a role in platelet aggregation, inflammatory response and assembly of extracellular matrix. THBS2 may be expressed by activated endothelial cells and act as a potent angiogenesis inhibitor. Its expression is induced by NF-kB depletion. On the other side THBS2 can enhance Notch signaling. It was shown to be associated with the pathogenesis of coronary disease and myocardial infarction. Growing body of evidence shows that THBS2 could play a role in the development and progression of different neoplasms.

Methods: Microarray analysis of 99 ovarian cancer samples was performed using Affymetrix HGU 133 Plus arrays. Real-time PCR was done with AGI PRISM 7900 HT System. THBS2 coding sequence was reverse transcribed from human fibroblasts and cloned in two fragments using TOPO vector than ligated into the pLNCX2 vector. Competent bacteria were transformed using several modifications of chemical method and electroporation.

Results: In our microarray study THBS2 gene was found as one of the potential prognostic markers correlated with the survival rate of ovarian cancer patients. These results were confirmed by real-time PCR. In the next step we decided to check the role of the THBS2 using the in vitro model. We planned to evaluate whether THBS2 may influence chemotheraphy resistance, ability to undergo apoptosis and motility of cancer cells. We checked 5 ovarian cancer cell lines for the expression of THBS2. Only one line was able to synthesize this protein. Thus we aimed to modify 4 other cell lines to obtain overexpression of THBS2. Than the original and modified cell lines could be analyzed by different in vitro tests. However, so far our attempts to clone THBS2 cDNA in pLNCX2 vector were unsuccessful.

Discussion: As a possible reasons for the cloning failure we excluded inefficient ligation and recombinant vector size (possibly to large). It seems that the last option is that THBS2 protein is toxic for bacteria. We are currently investigating this possibility. We would greatly appreciate the discussion and feedback from the conference attendants on how to overcome this problem and successfully clone the THBS2 gene.

Conclusion: Preliminary results indicate that THBS2 may play an important role in ovarian cancer progression, however this must be further confirmed.

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**[224] Heat Shock Shock Transfection Factor 1 (HSF1) Enhances Mobility of Mouse Melanoma B16(F10) Cells**

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Introduction: Heat Shock Transfection Factor 1 (HSF1) is a main regulator of the heat (stress) response. It activates heat shock genes, which encode heat shock proteins (HSPs). HSPs function as molecular chaperones by folding proteins during cellular stress. Beyond the classical induction of HSPs, HSF1 binds to broad array of non-HSP genes, associated with cell signaling, cytoplasmic organization and development. The obtained results suggest that this activity of HSF1 seems to be important for its role in carcinogenesis.

Materials and Methods: To study the role of HSF1 in tumor growth, we have constructed a model of mouse B16F10 melanoma cells with an overexpression
of constituively active, human HSF1, with deletion of regulatory domain (amino acids 221–315, HSF1ΔDRD). Simultaneously we have constructed cells with down-regulation of HSF1 expression using shRNA particles specific for 3'UTR and coding sequences of mouse HSF1.

**Results and Discussion:** The expression of HSF1ΔDRD was confirmed in transfected on mRNA and protein level. Expression of several inducible Hsp genes (Hsp1, Hsp2, Hspa1) was increased in cells expressing HSF1ΔDRD. The decreased expression (up to 50–70%) of HSF1 and several inducible Hsp genes (Hsp1, Hsp2, Hspa1) was detected in B16F10 cells with HSF1 knockdown. We have found that expression of constitutively active HSF1, enhanced anchorage independent growth of B16F10, while B16F10 cells with down-regulated expression of HSF1 has the same ability to form colonies in soft agar as nonmodiﬁed cells. Using Boyden chamber assay we showed that B16F10 expressing HSF1ΔDRD had higher ability to migrate. To determine the expression proﬁle of genes associated with cell motility, speciﬁc RT²PCR array was used. We found that expression of several genes (Vinc1, Cap1, Pk1b2) was decreased in B16F10 cells expressing HSF1ΔDRD.

Conclusions: We can conclude that HSF1 activation might support cell mobility and enhance tumor metastasis.

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cell lines as well as in the murine pancreatic carcinoma samples using quantitative real-time PCR. On protein level the expression of RAR and RXR was evaluated with immunohistochemistry. As differentiation markers cytokeratin 7 and carbonic anhydrase II were analyzed by immunocytochemistry. To determine epithelial-mesenchymal transition vimentin expression was assessed with immunocytochemistry. Cytotoxicity of retinoic acid was tested with the MTT assay and the effects on proliferation with the BRDU assay.

Results: On protein level RAR α and β is significantly lower expressed in seven out of nine pancreatic tumor cell lines compared to healthy cells. No difference in the expression occurred between primary tumor and metastatic cell lines on RNA and protein level. Retinoic acid treatment of pancreatic cancer cell lines with high and low expression of RAR showed neither cytotoxic, nor antiproliferative, nor differentiating effects. A negative correlation between vimentin expression and RAR α and β expression was found. In the orthologous system of murine pancreatic cancer RAR α and β as well as RXR α and β is significantly lower expressed in tumor samples compared to healthy pancreas at RNA level.

Conclusions: Our results show that malignant transformed pancreatic cells express less retinoid receptors than their healthy counterparts. Mesenchymal transition leads to decreased RAR α and β expression. It is tempting to speculate that the decreased retinoid receptor expression could play an important role in pancreatic cancer and it would be a reason for the negative clinical results of pancreatic carcinoma treatment with retinoids.

Identification of DNA Co-segregation During Cell Division in Breast Cancer Cell Lines

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Background: The breast cancer stem cell paradigm proposes the existence of subpopulations of cells within tumors responsible of driving disease progression given their capability of self-renew, indefinite proliferation and intrinsic resistance to drugs. Several antigenic markers have been used to identify, isolate and characterize cells with stem-like properties in breast cancer. However the inconsistency in the results obtained based on different cell surface markers, and the technical limitations of the models used to validate them claims for the design of less unbiased methods for cancer stem-like cells identification. We have exploited the stem cell property of DNA co-segregation during cell division to functionally identify putative breast cancer stem-like cells in luminal and basal breast cancer cell lines.

Material and Methods: We have used bromodeoxyuridine (BrDU) pulse-chase assays and the AMNIS system that combines flow cytometry with quantitative image analysis to identify cells that co-segregate their template DNA during mitoses.

Results: The asymmetric distribution of BrDU signal in phospho-histone H3+ and propidium iodide+ cells suggest that a small fraction of breast cancer cells co-segregate DNA during division, reminiscent of the process used by normal stem cells to maintain an immortal strain of DNA. We observed different ratios of DNA co-segregation between luminal and basal cell lines, correlating asymmetric division index with different cellular phenotypes.

Conclusions: Our results highlight the relevance of using functional approaches to identify putative tumor stem cells, and suggest that identifying and characterizing cells that divide asymmetrically could provide insight into the processes of tumor initiation, progression and chemoresistance in breast cancer.

Caveolin-1 Promotes Angiogenesis in Ewing’s Sarcoma Through EphA2 Signaling

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Introduction: Developing new blood vessels from preexisting capillaries is essential for tumor progression and metastasis. Caveolin-1 (CAV1) regulates multiple processes in tumor development including cellular transformation, tumor growth, multidrug resistance, angiogenesis and metastasis. Implication of CAV1 in angiogenesis of Ewing’s sarcoma (ESFT) has not been studied. Growth factors and ephrin (Eph) families are emerging as mediators of angiogenesis. In the present study we analyzed the hypothesis that CAV1 promotes angiogenesis and induces ESFT cell migration by activating EphA2 signaling.

Materials and Methods: Human ES cell lines were used in all experiments. Expression of CAV1 and EphA2 was analyzed using Western Blot and immunohistochemistry. Interaction between both proteins was analyzed by Immunofluorescence, confocal microscopy and Immuno-preparation assays. Total RNA analysis was performed by reverse transcription-PCR (RT-PCR). Pork aortic endothelial cells (PAEC) were used for endothelial migration.

Results and Discussion: We established low CAV1 expressing models by knocking down CAV1 in TC71, RDES, SKES1 and A673 cells by stably transfecting validated shRNA constructs. We observed a decrease in tumor volume from the CAV1 knocked down clones when compared with controls. This correlated with a reduction in microvascular density (MVD) and higher levels of necrosis. Tumor cell secrete factors that promote the growth and the migration of endothelial cells to the tumor. Thus, conditioned media from CAV1 knocked down cells showed reduced capability to promote migration of endothelial cells with no changes in proliferation. Different pro-angiogenic factors were analyzed by RT-PCR in the models and, downregulation of bFGF was observed in all four. Results suggested that CAV1 was indirectly affecting bFGF expression. The implication of bFGF in endothelial cell migration was confirmed using a neutralizing antibody for bFGF as well as a recombinant protein. Media obtained from control cells treated with the neutralizing antibody considerably reduced endothelial cell migration, while media from knocked down cells supplemented with the recombinant protein significantly increased migration. CAV1 has been shown to function as an activator of EphA2 signaling in endothelial cells. EphA2 expression was observed in ESFT cell lines and tumor samples. Moreover, we observed constitutive phosphorylation of the receptor. CAV1 knocked down cells showed a reduction in EphA2 phosphorylation as well as a displacement from the membrane to the cytoplasm. Furthermore, we showed that the interaction between CAV1 and EphA2 was necessary for Eph-mediated signaling.

Conclusion: EphA2 interacts with CAV1, thereby stimulating the production of bFGF, which in turn participates in the promotion of endothelial cell migration toward tumors.

Patterns of Tumor Evolution in High-grade Ovarian Adenocarcinoma

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Background: High grade serous ovarian cancer (HGS-OVCA) is characterized by a 25% rate of recurrence within six months after the end of treatment. Patient’s tumors rapidly develop resistance to the frontline platinum chemotherapies. As a result, the five-year survival rate is only 30%. To gain insight into the mechanisms used by tumor cells to evade the toxic effects of chemotherapy, we genetically characterized sixteen primary and recurrent HGS-OVCA tumors.

Methods: Sixteen matching HGS-OVCA primary and recurrent tumors were analyzed through exome sequencing (ten patients) and whole genome sequencing (six patients). All tumors were analyzed by DNA copy number, expression and methylation wide range of sensitivities. A high level of mutation was found in primary and recurrent tumors, with 24% of primary and 47% of recurrent tumors being platinum resistant (tumor progression within six months after last cycle of platinum administration).

Results: No specific mutations, copy number alterations (CNAs), methylation changes or expression changes were found to be associated with recurrence or the acquisition of platinum resistance; although the number of patients may not hold sufficient power to discover subtle correlations. All recurrent tumors acquired both somatic point mutations and CNAs. The number of acquired mutations and CNAs was positively correlated with the time elapsed between primary and recurrence. We modeled the clonal admixture in primary and recurrent tumors through analysis of mutation allele fractions and CNA profiles. All tumors were found to contain more than one clonal population. Interestingly, nine of sixteen recurrence samples harbored only a subset of the mutations and CNAs identified in the primary tumor. The analysis suggested that the clonal population in primary tumors was derived from a clonal population found in the primary tumor. In the remaining seven recurrent samples, in which all mutations and CNAs found at time of surgery were also identified in the recurrence, the dominant clonal population in the recurrence appeared to have progressively acquired major clonal events. A trend towards a difference in time to recurrence was observed between the two types of progression (p = 0.1).

Conclusion: Analysis of genomic alterations in primary and recurrent ovarian cancer tumors suggests that recurrences can be classified according to the details of the clonal structure of the tumor at time of surgery and may lend prognostic information to inform therapy. These findings shed light on the mechanism by which primary HGS-OVCA tumors re-develop and may lead to development of therapies that specifically address the clonal origin properties of recurrent tumors.
Background: The present study is to evaluate the effect of the overexpression of sialyl-Lewis x (SLe x) in Capan-1 pancreatic adenocarcinoma cell line, in regulating the function of membrane glycoproteins like a2b1 integrin, involved in cell adhesion to extracellular matrix proteins. Integrins can directly activate intracellular signalling processes, such as the ones involving focal adhesion kinase (FAK), and in cancer the up-regulation of integrins and FAK signalling promote migration and metastasis of tumour cells.

Material and Methods: Capan-1 is a pancreatic adenocarcinoma cell line with medium levels of endogenous a2,3-sialyltransferase ST3Gal III, and expresses SLe x. Transfected Capan-1 cells with ST3Gal III gene (C31) expressed the double of SLe x and almost four times less of a2,6-sialic acid structures. Flow cytometry analysis, and immunoprecipitation of a2b1 integrin and further blotting with anti-SLe x or Sambucus nigra lectin to detect a2,6 sialic acid, provided information of the a2b1 integrin glycosylation in both cell lines. In vitro adhesion and migration assays of Capan-1 compared to C31 were performed. FAK phosphorylation was also determined using specific antibodies against phosphorylated FAK tyrosine 397. Results: Transfected cells C31 had lower adhesion to collagen, main component of the extracellular matrix in pancreatic adenocarcinoma, while they showed increased migration through collagen compared to control cells Capan-1. Blocking Capan-1 cells with antibodies against integrin subunits showed that more than 90% of the adhesion to type 1 collagen takes place via a2b1 integrin. The a2b1 integrin in the transfected line C31 contained increased SLe x and decreased a2,6-sialic structures with respect to the one of Capan-1 cells. These glycosylation changes may contribute to a greater activation of the signaling pathway initiated by phosphorylation of FAK tyrosine 397, which was higher phosphorylated in C31 and would explain its greater migratory capacity. Conclusion: a2b1 integrin functionality is finely regulated by a shift in its sialic acid content and linkage. This strategy could supply the tumour cell with a powerful tool to regulate its adhesive and migratory capacities throughout the tumour progression steps.

233 Micro-managing Fibroblast Senescence – the Role of Small Non-coding RNA in the Senescence Associated Secretory Phenotype

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Introduction: Emerging evidence suggests that the acquisition of senescence associated secretory phenotype (SASP) by fibroblasts (as a result of ageing or in response to cytostatic stress) can enhance the proliferative and invasive phenotype of neighbouring cancer cells. This is thought to result from increased secretion of inflammatory cytokines, growth factors and extracellular matrix components by the senescent fibroblasts. The discrete molecular pathways that dictate acquisition of the SASP have yet to be elucidated. Here we examine the hypothesis that oral fibroblasts (OF) may develop a SASP associated secretory phenotype (SASP) that may in turn modulate the behaviour of cancer cells in the peripheral circulation. We hypothesize that SASP associated secretory phenotype (SASP) by fibroblasts may play a role in tumour progression and metastasis

Materials and Methods: OF were pulsed with hydrogen peroxide (H2O2) or cisplatin to induce premature senescence (PS), or allowed to reach senescence by SA-B-gal staining. Both p21cip1 and p16INK4a expression levels were determined by Western blot analysis. Normal oral fibroblasts were used as controls. RNA was extracted from OF using Trizol and reverse transcribed into cDNA using high capacity RNA-to-cDNA kit. Resulting cDNA was analyzed for miRNA expression using TaqMan miRNA assay. Results: Results show that oral fibroblasts have a pronounced expression of miR-146a, miR-141 and miR-222, which are known to modulate the SASP. miR-146a and miR-141 are involved in the regulation of cytokines and chemokines, while miR-222 is involved in the regulation of the PI3K/Akt/mTOR pathway, which is involved in SASP. Conclusion: This study shows that oral fibroblasts have a pronounced expression of miR-146a, miR-141 and miR-222, which are known to modulate the SASP. miR-146a and miR-141 are involved in the regulation of cytokines and chemokines, while miR-222 is involved in the regulation of the PI3K/Akt/mTOR pathway, which is involved in SASP. This study provides new insights into the role of miRNAs in the regulation of SASP and their potential as therapeutic targets in cancer.
Investigation of the Human Epidermal Growth Factor Receptor 2 (HER2) as a Biomarker for Chemorresistance in Breast Cancer

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Introduction: HER2 is overexpressed in a subset of breast cancer, and its expression is associated with poor prognosis. HER2 overexpression is a predictive marker for the efficacy of HER2-targeted therapies, such as lapatinib and trastuzumab. However, resistance to these therapies can occur due to the development of secondary mechanisms that confer resistance. One such mechanism is the suppression of the EMT program by HER2 inhibitors, which may lead to the reexpression of EMT markers.

Materials and Methods: We treated breast cancer cell lines with lapatinib and trastuzumab, and then assessed the expression of EMT markers using immunofluorescence and western blotting.

Results: We observed an increase in the expression of EMT markers such as ZEB1 and ZEB2 in HER2-inhibited cells compared to controls. This suggests that the HER2 inhibitors are promoting a return to an EMT-like phenotype.

Conclusion: Our findings support the hypothesis that HER2 inhibition may be promoting a reversion to an EMT-like state, which could be a mechanism of resistance to HER2-targeted therapy. Further research is needed to elucidate the underlying mechanisms and develop strategies to prevent this resistance.
[241] Tumor-derived Granulocyte-macrophage Colony Stimulating Factor is Responsible for Accumulation of Pro-invasive Microglia/macrophages and Glia Progression

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Introduction: Experimental and clinical studies show an important role of glioma-infiltrating macroglia in glioma progression. Macroglia are attracted by tumor-released molecules and instead of initiating anti-tumor responses, those cells support invasion, angiogenesis, extracellular matrix remodeling and immunosuppression indifferent types of tumors. In some experimental cancer, M-CSF/CSF-1 (macrophage-colony-stimulating factor/colony-stimulating factor 1) was reported as a crucial factor regulating infiltration and function of tumor-associated macroglia. We found that GL261 glioma cells express a marginal amount of csf-1 mRNA but the expression of csf-2/gm-csf (colony-stimulating factor 2/ granulocyte macrophage-colony-stimulating factor) is high in comparison to non-transformed astrocytes.

Material and Method: We generated EGFP/GL261glioma cells stably expressing GM-CSF specific or control shRNA. Silencing of GM-CSF expression was confirmed by qPCR and ELISA. Intracranial growth of control and GM-CSF depleted glioma cells, macroglia infiltration (Iba1 staining and determination of CD11b+CD45high and CD11b+CD45low populations in tumor bearing brains by flow cytometry), angiogenesis (vWFstaining) and animal survival were evaluated. Osteopetrotic mice (op/op) deficient in M-CSF were used to evaluate a role of M-CSF in glioma progression.

Results and Discussion: Silencing of gm-csf expression did not affect basal proliferator survival of cultured glioma cells. Intracranial gliomas depleted of GM-CSF showed highly reduced accumulation of Iba-1+ cells and significantly reduced proliferation or survival of cultured glioma cells. In intracranial gliomas depleted of GM-CSF, macroglia infiltration (Iba1 staining and determination of CD11b+CD45high and CD11b+CD45low populations in tumor bearing brains by flow cytometry), angiogenesis (vWFstaining) and animal survival were evaluated. Osteopetrotic mice (op/op) deficient in M-CSF were used to evaluate a role of M-CSF in glioma progression.

Conclusion: GM-CSF is responsible for tumor-driven accumulation of brain resident, peripheral macroglia and may be a new target for glioblastoma therapy.

[242] Identification of EPHA3 as a Candidate Tumor Suppressor in a Screen for Cellular Senescence


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Introduction: Premature engagement of a cellular senescence program is a common cellular response to prolonged oncogenic activation or loss of tumor suppressor function, acting as a physiological tumor suppression mechanism in premalignant human tumors. Characteristic features of cellular senescence comprise a permanent cell cycle arrest associated with cell morphological changes, such as cellular and nuclear flattening, and an increase in senescence-associated b-galactosidase (SA-b-Gal) activity. Senescence is also commonly triggered by activation of the (Eis)/p16INK4a/Rb tumor suppressor pathway.

Material and Methods: Since oncogene-induced senescence (OIS) is a commonly detected in cells containing intact cellular DDR and p16INK4a pathways, we reasoned that a siRNA screen in untransformed cells should identify putative tumor suppressor genes. We developed a screen applying cell morphology and p16INK4a assays to identify senescence-inducing kinase siRNAs, expecting to identify putative tumor suppressor pathways.

Results and Discussion: We successfully identified twelve kinases as novel regulators of cellular senescence. One such candidate was the EPHA3 receptor tyrosine kinase, a gene commonly mutated in human lung adenocarcinomas and colorectal cancers. We show that senescence in response to loss of EPHA3 expression is partially conferred by known actors of other signaling pathways, including ADAM23 and p53. Structural analysis of intracellular EPHA3 tumor-associated point mutations suggests molecular explanations on how mutations may impact on receptor kinase activity. To functionally show the consequence of somatic mutations, we express near-endogenous levels of GFP-tagged EPHA3 variants, which are correctly membrane localised and internalised upon ligand treatment. Importantly, we show that selected kinase domain point mutations cause a decrease in receptor expression level and/or normalised receptor tyrosine kinase (RTK) activity.

Conclusions: Our study describes a new strategy to uncover candidate tumor suppressors, and provides compelling evidence that EPHA3 mutations may promote tumorigenesis only when key senescence-inducing tumor suppressor pathways are inactive.

[243] Triple Negative Breast Carcinoma Cells Directly Contribute to Tumor Vasculature by Endothelial Differentiation Capability

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Introduction: Very aggressive at a clinical point of view, Triple Negative Breast Cancers (TNBCs) represent a question mark in BC biology. Here we explored TNBC vascular properties, particularly focusing on the capability of this subgroup of BC to create vascular structures.

Material and Method: Constituting TNBC harbord FFPE (Formalin-Fixed Paraffin Embedded) human TNBC specimens with anti-pan epithelial cytokeratins or anti-p53, and anti-CD31 or CD34 was evaluated by Immunofluorescence. Endothelial-like functional properties were investigated in vivo in BC cell lines seeded on Matrigel by Tube formation assay and in vivo by Confocal microscopy on frozen tumor sections. In vitro Vasculogetic mimicy (VM) and in vivo tumor growth were carried out impairing the main pathways involved in vessel formation by treatment with inhibitors (i.e. Sunnilinib and Bevacizumab) or siRNA-mediating silencing.

Results and Discussion: Immunofluorescence analysis indicated that only TNBCs show an endothelial-like phenotype. Moreover, analysis of VM revealed vascular tube channel formation in TNBC cell lines, whereas no vascular structures were generated by luminal or HER2-positive cells. In vitro VM also reflected a property of TNBC cells in vivo, where vascular lacunae were identified only in TNBC-derived tumors in comparison with a luminal xenograft model. To elucidate the mechanisms involved in the VM capability of TN tumor cell lines, we analyzed their VM in presence of Sunnilinib, targeting VEGFR, PDGFR and FGF, and Bevacizumab, against VEGFA. Whereas treatment with Sunnilinib completely abrogated the formation of vascular channels in vitro in all TNBC cell lines, the slight effect exerted by Bevacizumab suggested that this property is related to a VEGF-independent mechanism. In keeping with this evidence, siRNA-mediating silencing of the single receptors targeted by Sunnilinib revealed a crucial role exerted by FGFFR2 and PDGFR-α-mediated networks. Consistent with in vitro data, Sunnilinib induced tumor regression in TNBC xenograft models, whereas only a slight effect on tumor size was observed upon Bevacizumab treatment.

Conclusion: TNBC cells display an endothelial-like phenotype. This phenomenon is likely to be responsible of the aggressive nature of this BC subtype and the increased sensitivity to Sunnilinib seems to rely on the specific impairment of FGFFR2 and PDGFR-α-mediated pathways, new promising specific therapeutic targets.

[244] Intratumoral Societies – Subpopulations of Tumor Cells Expressing Different Levels of ADAM23 Seems Responsible for Different Aspects of Malignancy

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Background: Cancer evolve through the accumulation of genomic and epigenetic alterations that promote or progression towards malignancy. Epigenetic silencing of ADAM23 (A Desintegrin And Metalloprotease domain 23) gene is a recurrent event in a wide range of tumors, including pancreatic, breast, gastric and colorectal cancers. Hypermethylation of ADAM23 gene promoter has been associated with metastatic disease and poor overall survival in breast cancer patients (Verbisck et al. 2009). However the precise biological effects of ADAM23 downregulation during tumor progression is unknown. Here we sought to further clarify the role of ADAM23 in tumor progression through functional characterization of ADAM23 in vivo and in vitro.

Material and Methods: We stably knocked down ADAM23 expression in MDA-MB-231 and HTB-17 BC cell lines, expressing human ADAM23 in CMS5a, a mouse fibrosarcoma cell line. Cells transfected with empty vectors were used as controls in all comparisons. Invasion assays were performed embedding cell spheroids in a tri-dimensional collagen-type I matrix. Cell adhesion assay and migration assays were performed according to standard protocols. ADAM23 expression in primary tumors displaying ADAM23 promoter hypermethylation was analyzed by in situ hybridization (approved ethics committee).
RESULTS: We demonstrated that, in both the human and murine models, reduced levels of ADAM23 correlate with a 3-fold increase in the average speed of cell movement through collagen matrix. By contrast, in soft agar assays these fast invasive cells displayed a decrease of 50–70% in the colony sizes, with no changes in colony numbers. In vivo, ADAM23-positive human cells formed tumors (90%), whereas the tumorigenic rate of ADAM23-negative cells was only 33%. In situ hybridization of ADAM23 hypermethylated tumors revealed the co-existence of ADAM23 negative cell clusters and clusters expressing high levels of ADAM23.

Conclusions: Our results demonstrate that ADAM23 can act as a pleiotropic molecule by stimulating growth and tumorigenesis, but inhibiting invasion and suggest that the invasive phenotype is more likely to evolve after ADAM23 silencing in primary tumors. Finally, we hypothesize that the co-existence of tumor cell subpopulations expressing different levels of ADAM23 within primary tumors may contribute to malignancy given that association of both proliferative and invasive phenotypes facilitate accomplishment of metastatic cascade.

**245** DYRK1a Modulates the Self-renewal Capacity of Neural Stem Cells and Tumor Initiating Cells: Targeting the Achilles Heels of EGFR Addicted Glioblastomas

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Introduction: Glioblastomas (GBMs) are very aggressive primary brain tumors, being resistant to chemo and radio-therapy. There are important differences in the differentiation status inside a given GBM, with cells resembling normal neural stem cells (NSCs) on top of the cellular hierarchy. These so-called tumor initiating cells (TICs) can be grown in the same media used to expand NSCs, in the absence of serum. TIC and NSC also share many of the regulatory mechanisms. We have recently demonstrated that DYRK1a (Dual-specificity tyrosine(Y)-phosphorilation-Regulated Kinase 1a) controls the response of adult NSCs to EGF. Levels of DYRK1a activity determine receptor turnover, affecting stem cells expansion and maintenance in adult neurogenic niches so that in heterozygous mice there is a premature loss of NSCs. Interestingly EGFR is amplified, overexpressed or mutated in more than 50% of GBMs, being considered as one of the most important targets. However, most of the tyrosin-kinase inhibitors assayed in GBMs have shown poor therapeutic benefits.

Material and Methods: We have done the experiments in primary GBM lines derived from patients and established cell lines, all grown as neurospheres in the absence of serum. We have used a pharmacological inhibitor of DYRK1A kinase activity, Harmin, as well as RNA interference. We have tested the effect of DYRK1A blockade in vitro by analyzing EGFR stability, clonal growth, proliferation and TICs survival and we have checked the capacity to form intracraniach xenografts in nude mice. We have also performed a qRT-PCR expression analysis in a panel of gliomas of different grades.

Results and Discussion: Our results indicate that inhibition of DYRK1A kinase in GBM TIC-enriched cell lines promotes EGFR degradation, affecting proliferation and especially the survival of the TICs in those gliomas that express the receptor. Interestingly, Harmin induces a senescent phenotype in EGFR depending cell lines, without affecting the expression of stem cell markers. More importantly, pharmacological inhibition of DYRK1A or shRNA strategies, clearly impair tumor growth in vivo. Interestingly the analysis of a panel of gliomas shows that there is a strong correlation between DYRK1A and EGFR expression. In resume, our data suggest that recapitulation of EGF receptor stability is an essential oncogenic event in a percentage of GBMs, underlying the special nature of this type of tumors and the dependence of TICs (like NSCs) self-renewal on high levels of EGFR in the membrane.

**246** Rab25 Expression is Up-regulated in Head and Neck Cancer

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Introduction: Head and neck cancer (HNC) is the sixth most common type of cancer in the world. HNC includes larynx, oral cavity, pharynx, paranasal sinuses. The vast majority of HNC is related to exposure to carcinogens, including tobacco, alcohol, betel nut, and sexually transmitted viral pathogens such as human papilloma viruses. There has been a significant increase in knowledge regarding the molecular biology and genetics of head and neck squamous cell carcinoma. The fundamental genetic basis of all cancers is overexpression of oncogenes and/or silencing of tumor suppressor genes. The Rab25 gene has a homology to the Rab11 subfamily which includes Rab11a and Rab11b. Rab proteins, play an essential role in regulating signal transduction, and a diverse range of cellular processes, including differentiation, proliferation, vesicle transport, nuclear assembly and cytoskeleton formation. Rab25 has been functionally linked to tumor progression and the invasiveness of some epithelial cancers. The aim of this study was to investigate the role of Rab25 gene in the pathogenesis of head and neck cancer.

Materials and Methods: Expression of the Rab25 gene in head and neck cancer patients was investigated in 53 tumors and the corresponding non-cancerous tissue samples. Rab25 gene expression levels were analyzed by Quantitative Real Time reverse transcription-PCR (qRT-PCR). RTPCR was performed using the Light Cycler 480 II. The G6PDH gene was used as the housekeeping gene to standardize the quantification of mRNA levels. Statistical analyses were performed by the Paired t-Test.

Results and Discussion: The mean Rab25 expression levels for tumor and non-cancerous tissue samples were 1.06±0.12 and 1.16±0.11 respectively. The relative Rab25 expression ratio (Rab25/G6PDH) in the tumor tissues were significantly higher than in normal tissues (p = 0.032). Increased Rab25 expression was observed in 72% of the tumor tissue samples. Expression levels were also compared with clinicopathologic characteristics of the patients and was higher in females than males both for normal and tumor tissue samples (p = 0.025). The level of Rab25 expression was not associated with any other clinicopathologic parameters.

Conclusion: Increased Rab25 expression in tumor tissue indicate that the Rab25 gene may act as an oncogene in head and neck cancer progression.

**247** Alterations of Cellular and Molecular Patterns in Oxaliplatin Resistant Colorectal Adenocarcinoma Cell Lines

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Introduction: Oxaliplatin (L-OHP), a third generation platinum analogue is one of the leading treatments of colorectal cancers (CC), acting as bifunctional alkylating agent and forming of platinum-DNA adducts, which leads to the death of cancer cells. Despite of the improvements in the therapy of CC, partly due to targeted molecular therapies in addition to standard chemotherapy, resistance occurs in nearly all metastatic patients. The present study proposes to examine alterations of some cellular and molecular patterns in L-OHP resistant CC.

Material and Method: Chronic resistance to L-OHP was induced in Colo320 and HT-29 colorectal adenocarcinoma cell lines and morphology, cytotoxicity, DNA cross-links formation and gene expression profiles were assessed and compared with the parental cells. Morphology was assessed with light microscopy and cytotoxicity with MTS/PMS or MTT colorimetric cell proliferation assays. DNA cross-links formation was determined indirectly with alkaline comet assay by introducing single strand breaks via ionizing radiation. The reduction of these lesions in the cells treated with L-OHP, quantitatively reflected the cross-links induced by L-OHP. Gene expression profiles were determined with microarray technology; validation of the results was done by quantitative real-time PCR.

Results and Discussion: Morphology analyses revealed consistent shape and epithelial-to-mesenchymal transition in the resistant vs parental cells, suggesting the alteration of the cell adhesion complexes, through which cells acquire increased motility and invasiveness. Cytotoxicity tests demonstrated resistance to the drug in both cell lines, Colo320 being more sensitive than HT-29 (P <0.001). The induction of DNA cross-links was evident in both parental cell lines; in the resistant correspondent of HT-29, cross-links did not appear, unlike to Colo320, concordant with the cytotoxicity findings, indicating a higher sensitivity of Colo320 to this drug. Genes responsible for apoptosis inhibition and cellular proliferation were modulated, more disrupted functions and pathways being identified in HT-29 than in Colo320 cells (19 vs 11).

Conclusion: CC cell lines with identical origins adopted distinct cellular and molecular alterations during the resistance acquiring process and reacted differently to the subsequent treatments. Similar investigations on primary CC cells, correlated with the clinical evolution of the disease may help to elucidate the complex mechanism of chemoresistance and to identify potential targets for future therapies.

**248** Surveillance of Spontaneous Breast Cancer Metastases by TRAIL-Expressing CD34+ Cells in a Xenograft Model

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Introduction: Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in tumor cells while sparing normal cells. In hematological diseases, TRAIL has been demonstrated to be effective in eliminating small cell leukemia (SCL) sensitive cells by killing TRAIL expressed on the membrane of CD34+ cells (CD34+TRAIL) by soluble (s)TRAIL sensitive tumors and a partial overcoming of sTRAIL resistance by refractory ones. Since few breast cancer (BC) cell lines resulted sensitive to
Side Population in Human Glioblastoma Does Not Characterize Caveolin-1 Modulates Invasion of Ewing's Sarcoma Cells

The resultsonstromal-derivedaswellastumor-derivedendothelialcells agents constitutes an advantage or an impediment for drug delivery in the mouse model, enabling to separate tumor cells from host stromal cells, thereby allowing to clearly identify the cellular origin of the SP cells. Interestingly we found, that subcutaneous tumor bearing mice sacrificed 14 days after the last treatment were completely free of metastases, present instead in all control mice. Conclusion: Our results indicate that CD34-TRAIL treatment may give therapeutic benefits in BC, including increased apoptotic response, overcoming resistance to sTRAIL, and a significant anti-metastatic effect.

Side Population in Human Glioblastoma Does Not Characterize Cancer Stem Cell Populations and is Exclusively Stroma-derived

Progression of glioblastoma is proposed to be triggered by a cancer stem cell population, postulated to be responsible for tumor recurrence due to their resistance to radio- and chemotherapy. Resistance mechanisms may involve ATP-binding cassette (ABC) transporters on the cell membrane, which are responsible for drug efflux from the cell and may therefore represent putative cancer stem cell (CSC) markers. We investigated the presence of the Side Population (SP) phenotype, which is recognized by increased efflux of the Hoechst dye through ABC transporters, in human glioma biopsies as well as in intra-cranial xenograft models derived from human biopsy spheroids and human glioma cultures. We used a GFP expressing immunodeficient mice model, enabling to separate tumor cells from host stromal cells, thereby allowing to clearly identify the cellular origin of the SP cells. Interestingly we find that SP cells in human gliomas is uniquely stroma-derived, thus indicating that the SP phenotype is not a valid marker for glioma CSCs. Indeed the SP population present in glioma tissue is composed of endothelial and astrocytic cells, whereas neither stromal nor tumor-derived stem/progenitor populations in the adult brain possess efflux properties. We further determined the effect of anti-angiogenic treatment by bevacizumab on the efflux properties of stroma-derived endothelial cells in order to address the question whether normalization of the vasculature induced by anti-VEGF agents constitutes an advantage or an impediment for drug delivery in the brain. The results on stroma-derived as well as tumor-derived endothelial cells will be discussed.

Caveolin-1 Modulates Invasion of Ewing’s Sarcoma Cells

Independently of Tyr-14 Phosphorylation and Through the MAPK Pathway

Introduction: Ewing’s sarcoma is the second most common bone tumor in childhood. Such tumors have a characteristic gene translocation that gives rise to a fusion protein, most commonly EWS/FLI1. This fusion protein acts as an aberrant transcription factor regulating the expression of different target genes involved in the proliferation, maintenance and progression of the tumor. Our group described caveolin-1 (CAV1) as one of these target genes, demonstrating its role in the malignant phenotype of Ewing’s sarcoma. Moreover, we described its function promoting tumor metastasis through the regulation of matrix metalloploproteinases (MMPs) and 2 and 9. CAV1 seems to regulate indirectly MMP9 transcription. Thus, in the present study we will ascertain the mechanism through which CAV1 exerts its regulation.

Material and Methods: We used previously published models of CAV1 low expressing cells. By using a RTK signaling antibody array we analyzed signaling pathways affected by CAV1 downregulation. Results were validated by western blot. By Boyden chamber assays we measure cell migration and invasiveness in the presence of different pathway inhibitors. For MMP9 expression and activity we performed RT-PCR and zymography assays, respectively. Implication of CAV1 Tyr14 phosphorylation on the processes of migration and invasion was investigated by site directed mutagenesis generating a nonphosphorylatable mutant CAV1-Y14F that was stably transfected into A673 cells.

Results and Discussion: Analysis of the RTK signaling antibody arrays showed that our CAV1 knockdown models presented a reduction in sTRAIL phosphorylation. Ribosomal protein S6 forms part of the 40S subunit of the ribosome and is a target of the mTOR pathway. Nonetheless, neither p70-S6 kinase nor 4E-BP-1 was found altered in the absence of CAV1. Therefore, we analyzed another possible regulatory pathway. RpS6 can be phosphorylated as well by RSK1 in an ERK1/2-dependent manner. We showed that ERK1/2 phosphorylation is reduced in the absence of CAV1. In addition, we also showed a reduction on phospho-RSK1. Using a specific ERK1/2 inhibitor, U0126, RSK1 and rpS6 phosphorylation were reduced, the same as MMP9 expression and gelatinase activity suggesting that MMP9 transcription may be controlled through MAPK signaling. On the other hand, CAV1 phosphorylation in Tyr 14 is known to be a crucial regulatory event in Ewing’s sarcoma cells and we described that this regulation is independent of CAV1-Tyr14 phosphorylation.

Kallikrein-related Peptidase 4 and Prostate Cancer – an Extended Role in Tumour Progression

Background: Prostate cancer is a leading cause of male cancer-related deaths, worldwide. Kallikrein-related peptidase 4 (KLK4) is over-expressed in prostate cancer, versus benign disease, and induces prostate cancer cell proliferation, migration and invasion, in vitro, as well as an epithelial-to-mesenchymal transition. Identifying KLK4 substrates is essential to understand the molecular mechanism of action of this protease and to develop a more comprehensive understanding of its role in carcinogenesis. For a global, unbiased assessment of KLK4 substrates, we employed a proteomic approach to simultaneously screen for KLK4 substrates in PC-3 prostate cancer cell secretions.

Materials and Method: Proteins released from PC-3 cells were collected, concentrated and treated with active, recombinant KLK4, or an inactive control. In accordance with PROTein TOpography and Migration Analysis Platform (PROTOPMAP, [1]), protein digests were separated by SDS-PAGE and gel lanes sliced into horizontal sections. Proteins and/or protein fragments in each section were identified by in-gel trypsin digestion and LC-MS/MS, with those proteins detected in a gel section of lower molecular weight in the KLK4-digested lane, compared to the control, deemed potential KLK4 substrates.

Results: Of the 153 cell secreted proteins identified, 20 novel potential KLK4 substrates were detected, including other proteases, protease inhibitors, extracellular matrix proteins, cell adhesion molecules, cytokines and cell signalling molecules. The nature of many newly identified substrates confirmed the known role of KLK4 in prostate cancer cell proliferation, migration and invasion. More importantly, KLK4 also cleaved substrates involved in other crucial processes vital to the establishment of a cancerous pathology, including angiogenesis and immune modulation, potentially representing novel pathways perturbed by KLK4 proteolysis in prostate cancer.

Conclusion: This study has diversified the role of KLK4 in prostate cancer progression by application of a proteomic substrate screening platform. Evaluating the biological validity of the novel substrates identified herein will aid in elucidating pathways driving this disease, with the aim of targeting those pathways readily amenable to therapeutic intervention.

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Reference(s)

[252] Homeobox Proteins Recruit Polycomb Repressive Complexes to Repress INK4a
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Background: Cellular senescence represents a crucial barrier against malignant transformation. p16 is one of the key tumor suppressors controlling cell proliferation and senescence. p16 is encoded by INK4a, which is frequently altered in human cancers. Polycomb repressive complexes (PRC) play an important role in INK4a epigenetic silencing but how they are recruited to the INK4a promoter is not well understood. We identified HLX1 (H2.0-like homeobox 1) in a screen for senescence regulators.
Material and Methods: We combined cell proliferation assays with transcriptional and ChIP analysis in primary human fibroblasts to investigate the function of HLX1 in cellular senescence.
Results: We observed that HLX1 extends replicative lifespan and impedes oncogenic RAS-induced senescence. HLX1 inhibits INK4a expression by recruiting Polycomb repressive complexes to the INK4a promoter and also regulates other PRC target genes. PRC-dependent repression of INK4a expression is a conserved property among Homeobox proteins as exemplified by HOX6 (Homeobox A9).
Conclusion: Altogether these data provide evidence for a collaboration between Homeobox proteins and Polycomb repressive complexes in transcriptional regulation. This mechanism could have general relevance in development, senescence and cancer.
N. Popov and N. Martin contributed equally to this work.

[253] Role of NANOS Family Members in Tumor Progression
V. Andries1, K. Staes1, F. Van Roy1, VIB/UGent, Department for Molecular Biomedical Research, Zwijnaarde, Belgium
Introduction: In carcinomas a recurrent molecular event of high prognostic value is an inactivation of the oncogene E-cadherin. Differential gene expression analysis showed that the novel NANOS1 gene was downregulated in E-cadherin-transfected revertants of a breast cancer cell line (Strumane et al., 2006). In flies, NANOS is a maternal RNA-binding protein that acts together with Pumilio as a translational repressor. Genomic and transcriptomic analyses revealed the existence of three human Nanos homologs, hNanos-1 to-3. In human cancer, several lines of evidence indicate that hNanos members have the potential to confer malignancy. In vitro induction of hNanos-1 results in abrogation of cell aggregation, increased motility and transcriptomic changes in PRC target genes. hNanos-1 is a novel potential PRC partner of hNanos1. Hsp90, also called the cancer chaperone, is a molecular chaperone required for the stability and function of numerous oncogenic signaling proteins. It has been shown that Hsp90 plays a role in metastasis-related events and small-molecule Hsp90 inhibitors are currently being tested in clinical trials (reviewed by Tsutsumi, 2009). To investigate the role of hNanos proteins in normal and cancerous cells, we identified new hNanos interacting proteins by a pull-down experiment, followed by mass spectrometric identification (Kris Gevaert, Ghent).
Results and Discussion: We crossed our transgenic hNANOS-x mice with appropriate Cre lines in order to activate the transgenes in a tissue-specific manner. These mice are presently used in mouse tumor models, including a chemical hepatocarcinogenesis model and a transgenic prostate cancer model to investigate whether more malignant tumors are formed upon induction of hNanos-x expression in vitro, for instance, the liver or the prostate. In addition, pull-down experiments of the overexpressed MYC-targeted hNanos1 identified several new interacting proteins for hNanos1. Interestingly, we identified among them the heat-shock protein 90 (Hsp90) as a putative interaction partner of hNanos1. Hsp90, also called the cancer chaperone, is a molecular chaperone required for the stability and function of numerous oncogenic signaling proteins. It has been shown that Hsp90 plays a role in metastasis-related events and small-molecule Hsp90 inhibitors are currently being tested in clinical trials (reviewed by Tsutsumi, 2009). In further experiments, we want to scrutinize the functional implications of this Hsp90 binding as well as other interactions with hNanos proteins.
Conclusion: The ultimate goal of this project is to verify conclusively whether hNanos proteins are novel potent promoters of tumor formation or progression. The use of transgenic mouse models and the identification and characterization of interacting proteins of hNANOS-x will provide us more insight in this.

[254] HSP27 is Involved in Ovarian Cancer Cell Motility and Response to Paclitaxel
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Introduction: The Hepatocyte growth factor (HGF) triggers cell scattering, migration and invasiveness by binding its receptor encoded by the MET oncogene. In ovarian cancer cells lines, activation of the c-Met receptor by HGF results in activation of the p38 MAPK pathway and phosphorylation of the small Heat shock protein of 27 KDa (HSP27). HSP27 takes part in different biological functions such as cytoskeleton rearrangements and cell survival. HSP27 binds both actin and tubulin filaments and regulates their polymerization. HSP27 is often found over-expressed in epithelial tumors and might cause cancer cell resistance to chemotherapeutics. As ovarian cancer cells have elevated HSP27 levels, we investigated its role in mediating HGF-induced cell motility and invasion and its involvement in the cellular response to paclitaxel (a microtubule-stabilizing agent), a drug used in the treatment of ovarian carcinoma.
Material and Method: HSP27 was stably silenced with short hairpin-RNAs in the ovarian carcinoma cells SKOV-3. Scatter, motility and invasion assays were performed in vitro to assess its involvement in HGF-induced motility. Immunofluorescence analysis by confocal microscopy was used to study HSP27 silencing effects on HGF-triggered actin polymerization and on tubulin filaments upon paclitaxel treatment. HSP27 involvement in SKOV-3 response to paclitaxel was evaluated both in vitro (apoptosis assay) and in vivo (regression trial).
Results and Discussion: By the in vitro assays, it was evident that HSP27 silencing impaired SKOV-3 capability to scatter, move and invade in response to HGF. HSP27 knock-down prevented HGF-induced actin polymerization as shown by confocal microscopy images analysis. Besides, the apoptosis assay stressed that HSP27 silencing was able to sensitize SKOV-3 to paclitaxel by promoting cell death. This result was also confirmed by the in vivo experiments where HSP27-silenced tumors regressed with paclitaxel treatment at low doses while control cells were poorly affected. As observed by immunofluorescence, the effect of HSP27 knock-down in the response to paclitaxel was likely due to a massive reorganization of tubulin filaments, which were seen prevalently localized at the cell periphery.
Conclusion: The results showed that HSP27 was involved in the regulation of HGF-induced actin polymerization required for SKOV-3 motility and invasiveness. Furthermore, HSP27 over-expression was responsible for SKOV-3 resistance to treatment with paclitaxel as its silencing promotes cell sensitization to the chemotherapeutic. This effect was likely related to the more pronounced tubulin filament reorganization, highlighting a possible cooperation between HSP27 knock-down and paclitaxel in the dysregulation of cytoskeleton assembly. Evenly, HSP27 can be considered as a valuable molecular target for ovarian cancer treatment.

[255] The RNA-binding Protein RNPC1 Regulates Mdm2 Expression Via mRNA Stability
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Background: Mdm2, a target of p53 and an E3 ligase, is a primary negative regulator of p53. RNPC1, a RNA-binding protein, is a target of the p53 family, which is known to regulate p53 expression via translation and p53 expression via mRNA stability.
Materials and Methods: Both molecular and Cellular techniques, including transcriptional studies, gene expression, and protein stability and degradation, were used to define how Mdm2 is regulated by RNPC1 in vitro and in vivo.
Results: We found that over-expression of RNPC1 decreases, whereas knockdown of RNPC1 increases, Mdm2 expression independent of p53. We also showed that RNPC1 directly binds to a AU-rich element in Mdm2 3’UTR and promotes mRNA degradation.
Conclusion: We uncover a novel mechanism by which Mdm2 is regulated by RNPC1 via mRNA stability. We postulate that by modulating Mdm2 expression, RNPC1 might be explored as a marker and/or a target for cancer therapy.

[256] Evaluation of Rare Event Detection by Flow Cytometry for Cancer Prognosis
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Background: Metastatic disease is the major cause of cancer mortality. The formation of metastasis requires the migration of tumor cells from the primary sites to other organs. Therefore, the detection of circulating tumor cells (CTC) is target of several investigations once it could be useful to assess metastasis risk. In addition to CTC, studies on metastasis mechanisms in animal models suggest migration of hematopoietic bone marrow progenitor
cells (HBMP) to the lung and formation of pre-metastatic niches. These niches would be suitable environments for seeding of CTC and for the development of metastasis. Thus, we hypothesized that the patients with greater risk of developing metastasis are those not only with CTC but also with circulating HBMP. Therefore, the goal of this study was to determine the lower limit of detection of these rare populations in PB by flow cytometry.

Material and Methods: We tested the detection of rare populations in PB using a model composed by PB of healthy donors and the cell lines MCF-7 and HL-60, representing CTC and HBMPC, respectively. Each cell line was fluorescently labeled and mixed with whole PB in to achieve concentrations from 5% to 0.01% per 10^6 leukocytes. After lysis of red blood cells, the mixture was analyzed by flow cytometry, and at least 100,000 events per tube were acquired.

Results: Rare events such as 0.01% and 0.05% were successfully detected in our model. Using linear regression, we found that the determination coefficient between the expected number of target cells and the number of detected cells was R^2 = 0.99 to CTC and R^2 = 0.98 to HBMPC.

Conclusion: Detection of rare populations of cells in PB is feasible and may allow the investigation of CTC and HBMPC in cancer patients. The association of CTC and HBMPC and cancer progression would suggest that cancer is a systemic disease, even if it is not considered disseminated according to current understanding, once it interacts with the whole organism. This concept would provide the establishment of new approaches for the prevention of metastasis.

Role of Epidermal Growth Factor Receptor (EGFR) in Inflammation Induced Colorectal Cancers

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Colorectal cancer (CRC) is one of the major causes of mortality in the western world. Inflammatory bowel diseases such as Ulcerative Colitis, Crohn’s disease are associated with an increased risk of CRC suggesting that immune system activation contributes to tumor promotion. The Epidermal Growth Factor Receptor (EGFR), a member of the tyrosine kinase receptor superfamily, is known to be overexpressed in CRC, but its molecular functions in this disease are not fully known. This project employs a transgenic murine colitis model, wherein EGFR expression is specifically abrogated in the intestines of these mice using the Cre-loxP system. Azoxymethane (a genotoxic carcinogen) is administered to these mice, to produce random genetic alterations, followed by Dextran Sulphate Sodium Salt which causes chronic inflammation in distal and intermediate colon. Both treatments mimic the environment and reduction of colorectal tumors. Current knowledge shows that mice homozygous for the hypomorphic Egfr^v2 allele (EGFR^v2/v2) develop fewer tumors compared to wild type mice. However, tumor phenotype in a tissue specific deletion on EGFR is not yet known. The project aims at establishing a controlled role of EGFR in colon carcinogenesis. Our current observations show no difference in tumor incidence in mice lacking EGFR in intestinal epithelial cells (EGFR^v2/v2 mice) compared to controls (EGFR^+/+). However, when high fat food is administered during carcinogenesis, a higher tumor incidence in EGFR^v2/v2 mice is observed. Molecular analysis show higher inflammatory markers in EGFR^v2/v2 mice. Interestingly tumor incidence is reduced in mice lacking EGFR in the myeloid cell lineage.

Furthermore, in other intestinal tumor model mice, which are heterozygous mutants for the Adenomatosis Polyposis Coli gene Apc^min and spontaneously develop adenomatous polyps in the gut, the loss of EGFR leads to significant reduction in life span because of higher tumor burden. These observations suggest a tissue specific protective role of EGFR that is contrary to the current belief that EGFR is a tumor promoter. Further molecular investigations currently underway will reveal the mechanisms leading to the observed phenotype.

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Induction of Estrogen Receptor-negative Breast Cancer Cells, MDA-MB-231

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Introduction: Costunolide (C15H20O2) is a sesquiterpene lactone that was found to be a promising anticancer drug especially for ER-negative breast cancer.

Materials and Methods: The antiproliferative effects of phytoestrogens were tested by proliferation assays. Flow cytometry was performed to analyze the cell cycle. The effect of phytoestrogens on cell-signaling molecules was determined by Western blotting.

Results and Discussion: Genistein and quercetin inhibited the proliferation of MCF-7 and MCF-7 HER2 cells. This growth inhibition was accompanied with an increase of subG0/G1 apoptotic fractions. Genistein and quercetin induced extrinsic apoptosis pathway, up-regulating p53. Genistein and quercetin reduced the phosphorylation level of IкBα, and abrogated the nuclear translocation of p65 and its phosphorylation within the nucleus.

Conclusion: Genistein and quercetin exert their antiproliferative activity by inhibiting NFкB signaling. Phytoestrogens could be potential useful compounds to prevent or treat HER2-overexpressing breast cancer.

Phytoestrogens are known to prevent tumor induction. But their molecular mechanisms of action are largely unknown. This study aimed to examine the effect of genistein and quercetin on proliferation and apoptosis in HER2-overexpressing breast cancer cell lines.

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Conclusion: Genistein and quercetin exert their antiproliferative activity by inhibiting NFкB signaling. Phytoestrogens could be potential useful compounds to prevent or treat HER2-overexpressing breast cancer.

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Conclusion: genistein induces G2/M cell cycle arrest and apoptotic cell death via extrinsic pathway in MDA-MB-231 cells suggesting that it could be a promising anticancer drug especially for ER-negative breast cancer.
**Characterization of the Novel Proteins From Ornithine Decarboxylase Antizyme-1 Affinity Protein Complex**

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The human ornithine decarboxylase (ODC) antizyme-1 (AZ1) is a protein with 228 amino acids that participates in the crucial roles in molecular and cellular functions, including developmental regulation, cell cycle, proliferation, cell death, differentiation and tumorigenesis. AZ1 directly binds to ODC which promotes the dissociation of ODC homodimer, thus inhibiting its enzymatic activity then marks ODC undergoing the ubiquitin-independent posttranslational degradation by the 26S proteasome. Antizyme inhibitor (AZI) can displace ODC from the ODC-AZ complex and prevents ODC from being degraded by the 26S proteasome. Today, its role is seen in a considerably broader context, as AZ1 has been shown to affect the stability of several additional proteins such as cycin D1, Smad1 and Aurora-A. The aim of this study is to find the other AZ1 binding proteins and investigates its role for cellular molecular mechanism. There are now binding profile findings about cytoskeleton proteins. The total proteins from HeLa cells were pulled down with Ni-NTA affinity column and coimmunoprecipitated with AZ1 antibody in vitro. Mass spectrometry-based identification of the affinity component complexes were assayed again by polyclonal antibodies (PAGE) and in-gel digestion to detect novel interaction proteins. We validate the candidates by immoblotting in HeLa cells and further confirm the sub-cellular localization of the candidates and the specific affinity for AZ1 in vivo. We investigate the novel possible functional role of AZ1 in cells.

**Identification of Novel Cancer-associated Molecules**

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**Introduction:** Tumor metastasis is a major cause of cancer mortality. Despite significant advances in the treatment of primary tumors, the ability to predict metastatic behaviour of cancer, as well as detection and eradication of metastatic lesions, remains the greatest clinical challenge in oncology. Knowledge of the molecular mechanisms involved in metastatic spread and growth is needed to facilitate prognostic evaluation of individual patients and design therapies to inhibit the metastatic process.

In our study we aim at discovering metastasis-promoting molecules, revealing their functional role and preparing antibodies against these molecules.

**Material and Method:** We use two subclones of the MDA-MB-435 human melanoma cell line as a model for the metastatic spread of cancer. One cell clone metastasizes consistently to the lungs whereas the other, equally tumorigenic and capable of dissemination, fails to give rise to metastatic lesions at a secondary site in athymic mice. In order to find new metastasis-associated markers we screened these cell lines with phage-displayed single-chain variable fragment (scFv) antibody libraries. Specificity of the selected scFv-antibody clones was confirmed by Western blotting analysis. The major candidates were identified by immunoprecipitation followed by a mass-spectrometry analysis.

**Results and Discussion:** We found several scFv-antibody clones preferentially binding to the metastatic cells. The selected scFv-antibodies recognize antigens on the cell surface and some of them are able to internalize. We were able to identify the antigen for one of the selected antibodies. This protein is present in higher amounts on the surface of the metastatic cell clone compared to the non-metastatic clone. Currently, we study the role of the discovered KAZ2 membrane protein in tumor progression and metastasis.

**Conclusion:** Using the antibody-phage display approach we identified a novel membrane protein which is over-expressed by the highly metastatic cell line. Nothing is known about the function of this protein. We are going to perform in vitro and later also in vivo assays to uncover the role of this protein in normal development (zebra fish model) and cancer.

**Expression of Rho GDP Dissociation Inhibitor Correlates Positively With Lymph Node Metastasis in Colorectal Cancer**

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**Introduction:** The human colorectal cancer cell line SW620 was derived from primary tumor, whereas the SW620 line was derived from a colorectal metastatic focus in the same patient. We were able to identify the antigen for one of the selected antibodies. This expression using TMA, we identified a new metastasis biomarker protein candidate in colorectal cancer. We are currently studying the function and distribution of RhoGDI and its possible application as a cancer metastasis marker or therapeutic targets.

**Results and Discussion:** Proteins expressed at different levels in SW620 and SW480 were synthesized and identified using 2D-DIGE and MS. Expression profiles for these proteins, derived from antibody reactivity with TMDs, resulted in our identification of seven differentially expressed proteins in SW620. Changes in the expression pattern of one of these, Rho GDP dissociation inhibitor (RhoGDI), correlate positively with the clinical stage, tumor depth and degree of lymph node metastasis of this cancer. RhoGDI was also found to be expressed strongly in metastatic colon tumor cells.

**Conclusion:** By comparing the proteomes from two human colorectal cancer cell lines with different metastatic properties, and then confirming differential expression using TMA, we identified a new metastasis biomarker protein candidate in colorectal cancer. We are currently studying the function and distribution of RhoGDI and its possible application as a cancer metastasis marker or therapeutic targets.

**Lysyl Oxidase: a Potential Therapeutic Target for Clear Cell Renal Cell Carcinoma**

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**Introduction:** The copper-dependent lysyl oxidases (LOX), an extracellular matrix-modifying enzyme, plays a role in catalyse the lysine-derived cross-linking of collagen or elastin. LOX is expressed in tissues containing fibrillar collagen and/or elastic fibres. Abnormal LOX expression and enzymatic activity have been observed in various solid tumour cell lines and their corresponding tumour tissues including breast, lung, colon and oesophagus cancers. Kidney tumour represents 3% of all adult malignancies. The incidence and mortality rates of renal cell carcinoma (RCC) are steadily rising at a rate of approximately 2–3% worldwide. Clear cell RCC (ccRCC) is the most common type of RCC, representing 70–75% of all types. To date, the pathogenesis of ccRCC remains unclear. In this study, the expression of LOX in ccRCC and its roles related to cellular proliferation, migration, invasion and survival were investigated.

**Material and Methods:** The human tissues applied in this study were approved by National Research Ethics Service, NHS (REC reference number: 08/H1031/66). Gene expression profiles in ccRCC tissues were analyzed comparing to normal renal tissues. Further RT-PCR, western blotting and immunohistochemistry were used to validate the microarray data. Clear cell renal carcinoma cell line Caki-2 cells were cultured in a modified McCoy's 5a medium and treated with beta-aminopropionitrile (BAPN). The effects of reduced expression of LOX on cell proliferation, migration, invasion and apoptosis were assessed in vitro. The present study also demonstrates that inhibition of LOX expression in ccRCC cell line Caki-2 cells resulted in significant decrease of cell proliferation, migration and invasion in vitro, but had no effects on cell apoptosis.

**Conclusion:** LOX showed an important role in ccRCC cell proliferation, migration and invasion, therefore, LOX might be one of the potential therapeutic targets for ccRCC.

**Alpha-catenins Make the Switch**

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**Introduction:** In human epithelial carcinomas, progression to a malignant, invasive carcinoma is usually marked by loss of E-cadherin mediated adhesion leading to an epithelial-mesenchymal transition (EMT). However, loss of function of both α- and γ-catenin has been shown to contribute to mesenchymal phenotypes and metastatic behavior. Alpha-catenins are an essential part of the adherens junction complex between cells. Previous in vitro work showed that both αN- and γ-Catenin can restore cell-cell adhesion
and decrease hyperproliferation of carcinoma cells that lack α-catenin. In vivo models show that loss of α-catenin stimulates cell proliferation and growth-factor mediated signalling, while apoptosis is inhibited. We aim at unravelling in vivo the functional differences and possible overlaps between the various α-catenin isoforms. In particular, we wonder whether α-catenin induction can inhibit metastasis by α-catenin null carcinoma. Therefore, we will extensively analyze so-called protein switch mouse models in which α-catenin is conditionally replaced by γ-catenin in specific organs.

**Material and Methods:** We generated conditional transgenic mice to obtain controlled ectopic expression of murine γ-catenin in vivo. To this end, we targeted an α-catenin transgene into the widely expressed ROSA26 locus. In the configuration chosen, the expression of the transgene is dependent on Cre-recombinase mediated excision of a stop cassette. Crossing such mice with conditional α-catenin-KO mice results in an α-catenin switch in all Cre-expressing cells.

**Results and Discussion:** In skin, an α-catenin switch in basal layer keratinocytes by use of the K5-Cre model, shows a nearly complete functional overlap between α- and γ-catenin. Skin with α-catenin KO is characterized by keratinocyte hyperproliferation, pre-cancerous lesions and activation of the MAPK pathway. This lethal phenotype is completely rescued by ectopic α-catenin expression. Nonetheless, these transgenic mice show recurrent periods of partial baldness due to the inability of hairs to undergo correctly the cyclic transition from anagen to catagen. Whether this points at a functional discrepancy between α- and γ-catenin, or at insufficient α-catenin levels remains to be elucidated.

**Conclusion:** The results from the α-catenin switch model in murine skin provide proof-of-principle for the ongoing experiments in which the α-catenin protein switch will be assessed in several carcinoma models.

**[265]** Myristoylphorbol Acetate (MPA) Enhances Metastasis Via Up-regulation of Eph A2 Transcription Activity

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**Background:** Metastasis is the major death-cause of malignant tumor. A number of factors could regulate the tumor metastasis, including activators and inhibitors. One family of regulators is Eph receptor family. In contrast to their well-established role as antigen-presenting molecules are also important signaling receptors. The aim of our work is to understand the impact of signalling mediated by MHC class II molecules in addition to their well-established role as antigen-presenting molecules are also important signalling receptors. The results obtained showed an in vitro heterogeneity with respect to CAMs and HLA-DR proteins expression in melanoma progression, we compared in stimulated and unstimulated A375 melanoma cells the expression levels of class II and CAMs receptors and therefore in melanoma progressing to an in vivo metastatic state.

**Material and Methods:** Two MHC class II constitutive expressing human melanoma cell lines (A375 and HT144) with marked heterogeneity respect to invasion potential were stimulated for 24h, 48h and 72h with a specific anti-HLA-DR mAb (L243) that mimics the TCR interaction with class II molecules or left unstimulated. Therefore, the CAMs and HLA-DRs expression profile was analysed in total cell extracts. Exosomes secreted by stimulated and unstimulated melanoma cells were purified and analysed by western blot.

**Results and Discussion:** Within the hypothesis that MHC class II mediated signalling in constitutive expressing melanoma cells might have a role in melanoma progression, we compared in stimulated and unstimulated A375 and HT144 cell lines the expression levels of class II and CAMs receptors as well as the exosomes secreted. The results obtained showed an in vitro heterogeneity with respect to CAMs and HLA-DRs proteins expression in stimulated and unstimulated A375 and HT144 cells.

**Conclusions:** Our results provide some interesting information about the consequences of TCRs and MHC class II association that is mimed here by a specific anti-MHC class II antibody. In particular, this study helps to elucidate how the class II dependent signalling could affect in melanoma the extracellular matrix adhesion, the cell-to-cell interaction, the exosomes role on microenvironment and therefore the metastatic dissemination of melanoma.

**[266]** High ZNF217 Expression Level is a Novel Prognostic Marker in Breast Cancer and Deregulated ZNF217 Expression is Associated With Aggressive Phenotype

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**Background:** Cellular processes such as cell survival, growth, migration and gene expression are modulated by the interaction between cells and their microenvironment, CAMs (Cell adhesion molecules) that mediate adhesion, integrins and selectins, are the main receptors that facilitate the cells anchorage to the extracellular matrix and the cell-cell adhesion. The alteration of CAMs expression is related to metastatic progression of melanoma through the disruption of normal interactions between cells and cells with the matrix. Furthermore, the progression of melanoma is also associated to the constitutive expression of Major Histocompatibility Complex (MHC) class II molecules in melanoma cells. Indeed, the MHC class II molecules in addition to their well-established role as antigen-presenting molecules are also important signaling receptors. The aim of our work is to understand the impact of signalling mediated by MHC class II molecules in CAMs receptors expression and therefore in melanoma progressing to an in vivo metastatic state.

**Material and Methods:** We investigated the impact of ZNF217 expression level in vivo in melanoma progression. We compared in stimulated and unstimulated A375 melanoma cells the expression levels of class II and CAMs receptors as well as the exosomes secreted. The results obtained showed an in vitro heterogeneity with respect to CAMs and HLA-DRs proteins expression in stimulated and unstimulated A375 and HT144 cells.

**Conclusions:** Our results provide some interesting information about the consequences of TCRs and MHC class II association that is mimed here by a specific anti-MHC class II antibody. In particular, this study helps to elucidate how the class II dependent signalling could affect in melanoma the extracellular matrix adhesion, the cell-to-cell interaction, the exosomes role on microenvironment and therefore the metastatic dissemination of melanoma.

**[267]** Isolation and Characterization of Cancer Stem Cells in Solid Tumours

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**Background:** A growing consensus within the field of cancer research is the existence of rare tumour-initiating cells with similar properties to adult stem cells. These cells are termed ‘cancer stem cells’ (CSCs). Several markers associated with normal stem cells have been proposed as suitable markers for isolating CSCs, however, as yet, no universal marker of CSCs has been found. The study outlined here aims to determine the expression of different
CSC markers in unveal melanoma, cutaneous melanoma and prostate cancer cell lines and relate these to stem cell behaviour.

Materials and Methods: 3 unveal melanoma (UM) cell lines, 1 cutaneous melanoma (CM) cell line and 1 prostate cancer (CaP) cell line were used. Cells were seeded at clonal density to assess their ability to give rise to a stem cell hierarchy. Colonies were graded as holocenes, meroclines or paracleses with only holocenes being able to produce all three colony types. Cells were stained for expression of the stem cell markers CD44, CD133, aldehyde dehydrogenase (ALDH) and Nanog using flow cytometry. ALDH^+ cells were isolated using FACSAria. Proliferative and clonal assays were set up immediately following cell-sorting. Cells were counted 7 days post sort to ascertain proliferative rate. Colony numbers were assessed 10 days post sort.

Results and Discussion: The cell lines exhibited different capacities to generate a stem cell hierarchy. 2 UM cell lines and the CM cell line were unable to generate a stem cell hierarchy and all colonies had the same morphology and proliferative capacity. The CaP cell line PC3 and 1 UM cell line were shown to exhibit a stem cell hierarchy. CD44 was ubiquitously expressed in all cell lines whereas CD133 was not expressed at all across all 5 cell lines. Nanog expression varied from 0.5% to approximately 20% of cells in cell lines. ALDH^+ cells preferentially formed holocenes, the most primitive colony type, in cell lines. This suggests CD133 and Nanog may be associated with CSCs. Further studies are needed whether miR-106a and related signaling pathways could act as therapeutic targets of this disease.

Using Engineered Antibodies to Study the Limitations of Immunomagnetic Isolation of Tumor Cells

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Circulating tumour cells (CTCs) have recently attracted much interest in cancer biology as a way of studying metastasis. Characterization of CTCs can act as liquid biopsy thereby providing a non-invasive way of studying the tumour genotype, evolution and response to treatment. To date, the most widely used method of isolating CTCs has been immunomagnetic separation of cells using CellSearch™. However, very little is known about the fundamental features of cell isolation via antibody-targeted magnetic beads. How many target proteins per cell are required to enable cell isolation? How does the affinity of the antibody affect this limit? Can the presence of cytoskeletal inhibitors or activators affect the recovery of cells? Here, we test a clinically relevant saturated antibody fragment (Fab) against tyrosine kinase receptor HER2 expressed at high levels on a range of breast cancer cell lines. Comparing point mutants of the Fab with known kinetics, we establish a correlation between affinity and immunomagnetic isolating efficiency. We also show the effects of the cell membrane fluidity and cytoskeletal rigidity on the affinity of the magnetic bead to pull the cell out. Our results provide an insight into the molecular and cellular parameters governing immunomagnetic isolation and can be used to produce optimized sets of target receptors and biomarkers used for CTC isolation and other types of cell separation.

Alterations of miRNA Expression in Early-onset Turkish Colorectal Cancer Patients’ Tumor Tissues

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Introduction: Despite major efforts in diagnosis and treatment, colorectal cancer (CRC) is the second most common cancer in Turkey. In addition, the incidence of CRC has increased among early-onset patients. Survival rate from colorectal cancer differ dramatically according to metastasis development. To present, many tumor markers have been identified but none has provided a significant improvement over tumor stage as a prognostic indicator for cancer recurrence and metastasis for patients with early stage. Recent studies demonstrated that microRNAs (miRNAs) are one of the important set of biomarkers that can associate their specific expression profiles with cancer progress. Changes in miRNA expression are common features in CRC. However, depending on ethnic discrepancy, miRNA profiles may differ in tumors of patients. Because of miRNA changes among tumor subgroups of CRC have not been evaluated in Turkish population yet, the aim of this study was to clarify relevant alterations of miRNA expression associated with colorectal carcinogenesis and metastasis progression of early-onset Turkish patients.

Material and Method: We compared the expression profiles of 38 human miRNAs from 40-tumor with different stages and 8-non-tumor formalin-fixed paraffin-embedded (FFPE) CRC patients using miRNA custom PCR arrays (SA Biosciences). In addition, potential miRNA targets and associated pathways of significant miRNAs were predicted using miRWalk algorithm.

Results and Discussion: According to our findings, a group of miRNAs expression is significantly altered in different stages of Turkish CRC patients. The most interesting results came from miR-106a, frequently down regulated in high-grade CRC (P < 0.01). Using miRWalk, we identified miR-106a potentially involved in apoptosis, cell cycle and cell adhesion pathways.

Conclusion: Present results imply that expression patterns of microRNAs may be used as biomarkers of molecular classification of different stages of CRC. We suggest that, specifically, miR-106, which was shown to have association with poor survival of CRC patients in variable populations, is one of the biomarker of CRC’s metastatic potential in Turkish population. Even, further studies needed whether miR-106a and related signaling pathways could act as therapeutic targets of this disease.

Proliferation Networks Associated with Ki-67 and Progesterone Receptor Status in Invasive Breast Carcinomas

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Proliferation signatures have been reported in microarray datasets for invasive breast cancer. However, the signaling events associated with breast cancer proliferation are poorly understood. The aim of the present study was to explore the implications of the progesterone receptor (PR) and Ki-67 status, a tumor proliferation marker, in defining gene expression signatures in long-term follow-up patients. Large-scale gene expression analysis was performed in 34 cases (training set) using Whole Human Genome CodeLink bioarrays and in 54 samples (validation set) by real time qRT-PCR. Leave one out cross validation analysis detected a set of 83 genes differentially expressed (P < 0.001). Functional categorization based on Ingenuity Pathway Analysis (IPA) revealed the following main networks: cellular function and maintenance, cell cycle, cellular growth and proliferation involving 12 sequences; and 8 genes, potentially involved in transcription and cell division regulation, can contribute to increase the proliferative rate of breast carcinomas.

Supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Pesquisa (CNPq), Brazil.

Mis-7 Expression Depends on TP53 Mutational Status in Primary Serous Ovarian Cancer

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Background: Ovarian cancer remains an important clinical problem and long-term results of treatment are unsatisfactory. Mutations inactivating TP53 have been found in almost all types of human cancer still, even in malignant tumors with the wild-type TP53. The TP53-dependent tumor suppressor pathway is often a downstream target of the expressions of different cancer-related pathways. Our previous microRNA microarray analysis has revealed deregulated expression of miR-7 in primary serous ovarian cancer. miR-7 targets Bcl-2 and EGFR, known to play an important role in the pathogenesis of ovarian cancer.
Aim: We aimed to assess the expression of miR-7 and its targets Bcl-2 and EGFR in ovarian cancer and with different TP53 status.

Materials and Methods: Total RNA and gene expression was assessed by the qRT-PCR in normal human ovarian epithelium and in a panel of primary ovarian cancer samples with a different TP53 mutational status as well as in ovarian cancer cell lines that reflected the clinical samples with regard to their TP53 status.

Results: miR-7 was down-regulated in most of the clinical samples without TP53 mutations and overexpressed in the samples with TP53 mutations. We found a significant difference in miR-7 expression with regard to TP53 status (p < 0.01). Bcl-2 was up-regulated, while EGFR was down-regulated in most of the clinical samples and did not relate to the TP53 mutational status. There was no correlation between the expression of miR-7 and of its target genes. EGFR expression was decreased in ovarian cell lines. In ovarian cancer cell lines with TP53 missense mutations there was a reduced Bcl-2 expression, while an enhanced Bcl-2 expression was found in those with the wild-type TP53 and with TP53 null mutation.

Conclusions: 1. miR-7 seems to play an important role in ovarian cancer pathogenesis. 2. There seem to be a relationship between TP53 and miR-7. 3. In primary serious ovarian cancer the altered expression of miR-7 does not determine the levels of its targets genes, EGFR and Bcl-2.

Supported by the grant of Polish Ministry of Science and Higher Education (NN 401 050 138).

[274] Integrin α5β1 Plays a Critical Role in Resistance to Chemotherapy by Interfering With the P53 Pathway in High Grade Glioma


Introduction: Glioblastoma is the most aggressive and malignant form of brain tumour. Despite advances in standard therapy, including surgical resection followed by radiation and chemotherapy with temozolomide (TMZ), a significant number of patients will succumb to their disease. To address these challenges, significant research has been focused on the identification of new therapeutic targets. Integrin α5β1 is a receptor for the extracellular matrix and plays a role in cell migration and survival. We hypothesised that the down-regulation of integrin α5β1 in glioma cells could lead to increased sensitivity to chemotherapy. The aim of the study was comparison of the detecting methods of apoptotic cells of the study was comparison of the detecting methods of apoptotic cells.

Materials and Methods: We aimed to assess the expression of miR-7 and its targets Bcl-2 and EGFR in ovarian cancer and with different TP53 status.

Results: miR-7 was down-regulated in most of the clinical samples without TP53 mutations and overexpressed in the samples with TP53 mutations. We found a significant difference in miR-7 expression with regard to TP53 status (p < 0.01). Bcl-2 was up-regulated, while EGFR was down-regulated in most of the clinical samples and did not relate to the TP53 mutational status. There was no correlation between the expression of miR-7 and of its target genes. EGFR expression was decreased in ovarian cell lines. In ovarian cancer cell lines with TP53 missense mutations there was a reduced Bcl-2 expression, while an enhanced Bcl-2 expression was found in those with the wild-type TP53 and with TP53 null mutation.

Conclusions: 1. miR-7 seems to play an important role in ovarian cancer pathogenesis. 2. There seem to be a relationship between TP53 and miR-7. 3. In primary serious ovarian cancer the altered expression of miR-7 does not determine the levels of its targets genes, EGFR and Bcl-2.

Supported by the grant of Polish Ministry of Science and Higher Education (NN 401 050 138).

[275] Vav1 – the Oncogenic Switch

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Introduction: Since its discovery, research on the signal transducer Vav1 has cyclic under understanding its physiological function in the hematopoietic system and understanding how it is dysregulated as a truncated oncogene. Recent reports suggest that it is the wild-type (herein Vav1) form of Vav1, not its oncogenic form (oncVav1), which is involved in human cancers, such as neuroblastoma, pancreatic and lung cancer.

In the present study, we focused on understanding the possible role of Vav1 in breast cancer.

Materials and Methods: Immunohistochemistry, Western blotting, Real-Time PCR, soft agar foci formation, tunnel assay, immunofluorescence, GST-pull down, MTI and gene array analyses.

Results and Discussion: Immunohistochemical analysis of human breast cancer tissue array indicated that Vav1 is expressed in 44 of 70 (63%) tumors, mainly estrogen receptor positive cancers. These results highlight the potential importance of Vav1 in breast cancer. Next, we over-expressed Vav1 and oncVav1 in two breast cancer-derived lines, MCF-7 and AU565, which differ in their phenotype and cell-surface receptor expression. In both lines, over-expression of oncVav1 led to an increase in aggressive and enhanced proliferation of the cells. In contrast, over-expression of Vav1 led to opposing phenotypes in these two cell-lines; i.e., Vav1 led to reduced proliferation and enhanced cell death in MCF-7 cells, while it resulted in a similar proliferative effect to that of oncVav1 in AU565 cells. Gene expression microarray showed an increase in mRNA expression of proliferation-related genes in Vav1-expressing AU565 cells compared to a significant increase in apoptosis-related genes in Vav1-expressing MCF-7 cells. Experiments performed to assess whether expression of Vav1 in the transfected cells led to increased apoptosis, confirmed our genomics array analysis. Thus, Vav1-expressing AU565 cells are more aggressive and less prone to death compared to MCF-7 Vav1-expressing cells that are less aggressive and more apoptotic. Since AU565 cells contain a null p53 mutant, while MCF-7 cells express wild-type p53, we hypothesized that the Vav1 effect in these cell lines is p53-dependent. Silencing p53 in MCF-7 Vav1-expressing cells led to a marked decrease in apoptosis of these cells, thus erasing the Vav1 effect. These results demonstrate that Vav1 may function upstream to p53 in MCF-7 cells, but not in AU565 cells, in which it promotes proliferation.

Conclusion: Our results highlight for the first time the potential role of Vav1 as an oncogenic stress activator in cancer.

[277] Identification of Novel Therapeutic Targets Through an Integrated In Vitro and in Vivo Whole Genome ShRNA Screen in Glioma Stem Cells

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Anti-angiogenic therapy holds promise for the treatment of Glioblastoma (GBM), an aggressive brain tumor with dismal prognosis. However, so far overall patient survival is barely improved and tumors quickly develop resistance mechanisms towards anti-angiogenic treatment. In preclinical GBM models we have recently shown that the anti-angiogenic agent bevacizumab induces a significant reduction of blood flow in GBM tumors, which is accompanied by an increase in tumor invasiveness and hypoxia. Based on the hypothesis that GBM contain subpopulations of cells exhibiting stem cell properties (glioma stem cells) and are able to survive and thrive in a
hypoxic environment, we undertook an integrated molecular analysis of the glioma stem cells to identify novel key regulators of survival under hypoxia. A functional shRNA lethality screen with gene expression data from cultured cells, as well as transcriptomic data from patient GBM samples available from public databases, we defined a list of 56 candidate genes for in vivo validation. From the 56 genes, a pool of cell clones, each carrying a gene specific shRNA knockdown, was prepared for intracranial implantation in immunodeficient mice. The clones with genes essential for in vivo growth were expected to be lost during tumor development in vivo. Thirteen genes were found to be depleted in orthotopic tumors as determined by deep-sequencing. We have thus identified essential survival genes in glioma stem cells in vivo that represent novel molecular targets for drug development against highly resistant cells that cause tumor relapse.

Conclusions: CD133(+) cells had CSC-like properties in the cholangiocarcinoma cell line NCH421k using the human GIPZ lentiviral shRNAmmir library (Open MO59K stable knockdowns of Ku70 and Ku80 are being made to compare MIB7 and Ku80 proteins appear to play different roles in DSB end-joining, and which factors and clinical prognosis between CD133(+) patients and CD133(−) patients. By immunohisto pathological analysis of CD133, there was no significant difference in the clinicopathological factors and clinical prognosis between CD133(+) patients and CD133(−) patients. Conclusions: CD133(+) cells had CSC-like properties in the cholangiocarcinoma cell line from the mouse model. However, there was no relation between the clinical factors and CD133 expression. We could not identify the CSCs directly related to cancer therapy only by CD133 expression, but CD133 must be one of the key markers of CSCs in cholangiocarcinoma.

Conclusions: Microhomology-mediated double strand break (DSB) repair (MMEJ) is characterized by large DNA deletions and joining via ≥5-20bp microhomologies (MH). Its use is promoted by loss of the Ku70/Ku80 heterodimer. In a small panel of bladder tumours, we found that superficial tumours show mainly accurate non-homologous end-joining (NHEJ), while muscle invasive tumours (MIBTs) repair DSBs by MMEJ, show loss of p53 and Ku-DNA binding. We concluded that our system could be useful in determining the prognostic value of quiescent cancer stem cells in SCC. In addition, our molecular studies might help us to identify strategies to selectively eliminate this population of cancer stem cells which is commonly refractory to conventional chemotherapies that target highly proliferative cells.

Materials and Methods: To examine end-joining in bladder tumours, we used an in vitro end-joining assay. Cell-free protein extracts were prepared from tumour samples and added to linearized plasmid containing either compatible or incompatible ends in a ligation reaction. The ligation was PCR amplified using primers spanning the join, and resulting bands were cloned and Sanger sequenced. Extraction of the MO59K glioma cell line (which accurately joins both compatible and incompatible plasmid ends), were depleted of Ku70 or Ku80 by incubating with antibody and protein-A beads. Antibody-depleted supernatant was used on western blot and in the end-joining assay. Results: We expanded our tumour panel and confirmed the MIB7 and superficial tumour end-joining phenotypes. We will use EMSAs to investigate whether reduced Ku-DNA binding is due to competition by other proteins including PARP1, histone H1, p53 and MRE11. In MO59K cell extracts depleted of Ku70 or Ku80, we observed a reduction in end-joining accuracy of compatible (reduced from >99% to 65%) and incompatible ends (95% to 15%). The average deletion size and MH length differs for Ku70 (113.2bp deletion; 5.38bp MH) and Ku80 (205.1bp deletion; 1.67bp MH).

To examine if there is a critical threshold below which MMEJ takes over, MO59K stable knockdowns of Ku70 and Ku80 are being made to compare joining in wildtype cells and clones with different levels of knockdown. Conclusions: MIB7 display a MMEJ phenotype, associated with reduced Ku-DNA binding which may be due to competition from other proteins. Ku70 and Ku80 proteins appear to play different roles in DSB end-joining, and there may be a critical threshold determining NHEJ vs. MMEJ use.

Molecular and Functional Characterization of Quiescent and Proliferative Cancer Stem Cells in Human Squamous Cell Carcinoma in Vivo

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Introduction: Recent data support the existence of a population of Cancer Stem Cells (CSCs) in human solid cancers, including squamous cell carcinomas (SCC). So far, studies in SCC have identified CSCs capable of initiating tumor development, but little is known about their functional role. It has been recently shown that both human mammary carcinomas and mouse melanomas contain a quiescent subpopulation of cancer stem cells that are able to sustain tumor formation and are responsible of tumor relapse. This feature is of main interest because the standard chemotherapy is directed to eliminate actively proliferating cells, without affecting the slow-cycling population of the tumors. The aim of the project is to characterize the main pathways that are involved in maintaining the quiescent state of cancer stem cells in head and neck squamous cell carcinomas (HNSCC) in vivo.

Materials and Methods: To study the biology of HNSCC tumor initiating cells, we have developed a novel in vivo method that fully recapitulates the growth and development of human squamous cell carcinoma (HNSCC) in vivo. Using this transplant system we have developed means to detect and purify actively proliferating and long-term quiescent populations of cancer stem cells directly from the tumors and without any need to genetically modify them. We have combined transcriptome analysis and functional assays to determine the characteristics of each subpopulation of SCC cancer stem cells, and their in vivo significance.

Results and Discussion: Our results confirm that the population of human SCCs cancer stem cells is heterogeneous and can be predominantly subdivided into actively proliferating and long term slow cycling subpopulations. The analysis of the molecular signature of the quiescent versus active cancer stem cell populations revealed that genes associated with invasion, chemotherapy and EMT are up-regulated in the slow cycling population of the tumor, whereas genes associated with cell cycle progression and genomic instability are over-represented in the active population. In addition, we are identifying cell surface markers preferentially expressed in the quiescent cancer stem cells that might help us isolate and target them directly from human SCC samples.

Conclusion: We described here a new methodology to study the behavior of human squamous cell carcinoma in vivo in a setting that is more similar to the patient that does not the classical subcutaneous transplantation. We used this novel system to purify quiescent and active cancer stem cells to perform molecular and functional analyses. We conclude that our system could be useful in determining the prognostic value of quiescent cancer stem cells in SCC. In addition, our molecular studies might help us to identify strategies to selectively eliminate this population of cancer stem cells which is commonly refractory to conventional chemotherapies that target highly proliferative cells.
Background: The splice variant of HER2 lacking exon 16 (Δ16HER2) is expressed in about 90% of HER2-overexpressing primary breast cancers (BCs) and its transcript accounts for the 4–9% of total HER2. This isoform lacks expressed in about 90% of HER2-overexpressing primary breast cancers.

Materials and Methods: Immunophenotype of Δ16HER2-overexpressing tumor cells was analyzed by flow cytometry (FC) and immunocytochemistry (ICC). Δ16HER2 downstream signaling was evaluated by western blot (WB) of both tumor cell lines and biopsies, which were also examined by immunohistochemistry.

Results: Analyses carried out in mammary tumors indicated that they are constituted by two different cell components: a classic polygonal epithelial one, overexpressing both HER2 and estrogen receptor, and a smaller spindle-shaped one, weakly-positive or negative for both receptors. Distinct receptor expression is composed by monochromatic and classic epithelial, Δ16HER2-overexpressing tumor cells. From the disaggregation of mammary tumor biopsies followed by immunomagnetic separation with an anti-HER2 monoclonal antibody (MAb), we obtained some Δ16HER2-overexpressing cell lines. ICC and FC revealed that Δ16HER2-positive tumor cells expressed conventional epithelial markers as EpCAM, E-cadherin and cytokeratin 14 and also myoepithelial markers as vimentin, N-cadherin and cytokeratin 14 in a low percentage of tumor cells. WB analyses indicated that Δ16HER2 forms stable homodimers both on tumor cell lines and lesions activating multiple downstream signaling pathways as Src, Akt and MAPK. A lower binding of trastuzumab compared to control anti-HER2 MAb was observed on Δ16HER2-overexpressing tumor cell lines, confirming previously reported data on trastuzumab.

Conclusions: Δ16HER2 transgenic mice appear to be a preclinical model of luminal B BC known to have a worse disease course compared to luminal A BC. In addition, Δ16HER2 tumors are characterized by a late onset of epithelial-mesenchymal transition that is considered a characteristic of aggressive cancers. Further analyses are ongoing to deeply characterize our model and the role of Δ16HER2 in response to biodrug susceptibility.

Increased Activation of TAK1/NF-kappaB Activity is Associated With the Aggressiveness of Ovarian Cancers

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Introduction: TGF-beta-activated kinase-1 (TAK1), a mitogen-activated protein kinase, exerts a variety of biophysiological functions through governing numerous downstream signalings such as TGF beta, JNK and NFκB depending on the cell types and stimuli. Emerging evidences have revealed that TAK1 is significantly involved in human cancer progression. However, the functional roles and molecular mechanisms of TAK1 in ovarian cancers remain totally obscure.

Materials and Methods: Quantitative RT-PCR (Q-PCR) and Immunohistochemistry (IHC) analyses were used to evaluate TAK1 expression level in human ovarian cancer samples. Stable overexpressing TAK1 was generated by transfection of pCMV-HA:TAK and pCMV-FLAG-MAT-Tag-1-TAK1 in 16HER2-overexpressing tumor cell lines. The overexpressed data was analyzed by western blotting. Western blotting was performed to study the molecular mechanism of TAK1 by using antibodies p-TAK1 (Ser412, Thr184/187), p-IKK, p-AMPK and β-actin.

Results and Discussion: O-Q-PCR results showed that TAK1 was significantly upregulated (p = 0.005) in clinical ovarian cancer samples (n = 87) as compared with normal controls (n = 47). IHC analysis on an ovarian cancer tissue array further validated that TAK1 was overexpressed in ovarian cancer samples. Importantly, clinical-pathological correlation analysis revealed that the overexpressed TAK1 was significantly associated with high-grade (P = 0.08), lymph node and distant metastasis (P = 0.022), as well as a tendentially increased stage ovarian cancer independently. Increased expression of TAK1 was able to augment cell proliferation, colony formation, anchorage independent growth and migration/invasion in ovarian cancer cells. Conversely, depletion of endogenous TAK1 in ovarian cancer cells noticeably abrogated these tumorigenic capacities. Similar results were observed by specific inhibition of TAK1 activity by (S2)-T-Oxozeaenol. In addition, co-treatment of (S2)-Oxozeaenol and chemosensitizer such as paclitaxel or vincristine abrogated these tumorigenic capacities. Moreover, our data showed that there was an increase of Ser412 but not Thr184/187 phosphorylation of TAK1 in ovarian cancer cells. Importantly, the increased phosphorylated TAK1 was accompanied by elevation of expression of phospho-IKK, indicating the activated TAK1 may upregulate NFκB signaling activity in ovarian cancer cells.

Conclusion: Our findings suggest that the aberrant activation of TAK1 is correlated with ovarian cancer cell growth and invasion in a dose-dependent manner. Further biochemical studies in delineating the functional importance of TAK1 phosphorylation at Ser412 in governing NFκB signaling activity are warranted.

Linear Model Analysis Allows the Identification of De Novo and Acquired Resistance Related Genes

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Introduction: Ovarian cancer is the 5th most frequent cause of cancer death in women worldwide. Current standard treatments include debulking surgery as well as combination treatment with platinum and taxanes. Despite treatment improvements, 20–30% of patients never have a clinical remission and the majority of women will eventually relapse with incurable disease. Hence, aim of this study was to identify differently expressed genes related to acquired or de novo resistance. Therefore, a linear model was used to analyze the interaction between treatment and chemosensitivity in tumors derived from the cisplatin-sensitive human ovarian cancer cell line A2780 and its cisplatin-resistant derivative A2780cis. These genes might represent potential therapeutic targets for a 2nd line therapy in patients with resistant ovarian cancer.

Materials and Methods: A2780 and A2780cis cells were inoculated subcutaneously onto SCID mice and tumor growth was monitored. The tumors were treated with carboplatin (75 mg/kg; Q1Dox) or vehicle, and harvested 24h after last injection. RNA isolated from A2780 and A2780cis tumors and from monoclonal cell lines was hybridized to Illumina HT12v3 arrays. Differentially expressed genes were subjected to pathway analysis and further analyzed by RT-PCR. To verify potentially activated resistance-related signaling pathways, a human phospho-kinase protein array and TNF-alpha or IL-6-ELISA was performed.

Results and Discussion: Using the linear model analysis we identified 74 genes potentially involved in the development of de novo and acquired resistance, of which nine genes were further analyzed using pathway enrichment analysis. Of these nine genes, GDF15, DRAM1, P4HA2 and LMNA, were all shown to be linked to TNFR / AP-1 / ATF / TP53 signaling. Interestingly, in sensitive tumors basal TNF-alpha and IL-6 protein levels are higher than in resistant tumors and reduced upon carboplatin treatment. In contrast, no treatment-induced alteration of these cytokines was found in resistant tumors. This was associated with an increased expression of pro-apoptotic genes following treatment in sensitive tumors, whereas in resistant tumors no up-regulation occurred in a much lesser extent. This observation coincided with a reduced Caspase3/7 induction during carboplatin treatment in A2780cis as compared to A2780cis in vitro. Additionally, in sensitive tumors protein phosphorylation of p53 was downregulated, but remained unchanged in resistant tumors following treatment. In contrast, strong basal phosphorylation of the anti-apoptotic Akt kinase was found in resistant tumors, even under carboplatin treatment, but not in sensitive tumors.

Conclusion: We report for the first time that linear model analysis can identify potential factors involved in acquired and de novo resistance. If causality can be proven, targeting these factors might represent a novel treatment strategy for chemoresistant ovarian cancer patients.

Slug Controls P-cadherin and Regulates Stem/progenitor Cell Dynamics During Mammary Gland Morphogenesis

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Progression of breast cancer appears to be related to a partial dedifferentiation of epithelial cell phenotype. This phenomenon is known as epithelial-mesenchymal transition (EMT). TGF-β is now acknowledged as a key player in integrating plasticity and motility during cancer progression. A set of genes, called ‘EMT master genes’ has been characterized including transcription factors such as Snail, Twist or Zeb. Slug, a Snail family member, is described to be involved in early differentiation in skin and hematopoietic system. In mice mammary gland, we found that Slug is localized in about 10% of basal cells, and co-expressed with P-cadherin, C5K and CD49f (mammary stem/progenitor cell markers), in single cells, even in budding ducts. Functionally, Slug gain-of-function led to a decrease of cell growth and colony formation. Thereby, we hypothesize that Slug is involved in the morphogenesis of the mammary gland by the regulation of progenitor cells, and could, in a pathological context, determine the evolution of certain carcinomas and the
emergence of metastasis. To test this hypothesis, two approaches were used: in vivo and in vitro experiments. Among them, we realized orthotopic grafts of mammary glands from normal or Slug-deficient mice in each side of the same mouse to have the same environment, and clonal mammosphere culture to obtain an enrichment of stem/progenitor cells.

Orthotopically injected Slug-linker to podoepithelial impairment reflected by an over-branching, suggesting premature differentiation. A similar pattern was observed previously in P-cadherin deficient mice. Thereafter, additional gain or loss experiments demonstrated that Slug controls directly P-cadherin expression, as shown in E-boxes in P-cadherin promoter. This also showed that P-cadherin mediates Slug effects on cell migration. Slug deficit in primary or immortalized mammary epithelial cells decreases the growth of primary clonal mammospheres, and totally suppress secondary clonal mammospheres. These results indicate that Slug is involved in cells survival, apoptosis resistance, proliferation, self-renewal and maintenance of a subpopulation of progenitors cells. Moreover, we showed that Slug regulates in vivo some genes involved in luminal differentiation pathways such as CD24, GATA3 and ER.

Thus, we found that Slug controls maintenance and mobilization of a stem/progenitor cellular sub-fraction, playing a role in an early differentiation stage of mammary gland morphogenesis. Because the basal-like breast carcinomas involve poorly differentiated cells and overexpress Slug, this new Slug/P-cadherin pathway should provide new therapeutic approaches.

**Antiproliferative Activity of F14512, a Novel Polyamine-vectorized Drug, on Resistant Cancer Cells**


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**Background:** F14512 is a novel vectorized topoisomerase II inhibitor currently in phase I clinical study in AML. Briefly, F14512 is a polyamine-epipodophyllotoxin conjugate, that specifically targets topoisomerase II in Polyamine Transport System positive cells. This targeted therapy established a large therapeutic index. One of the most important limitations in chemotherapy treatment is acquired or innate drug resistance.

**Material and Methods:** To investigate the mechanisms by which cancer cells may escape from cell death induced by F14512, we established A549 NSCLC resistant cell lines which were obtained after 46 passages and 32 treatments of F14512 with doses ranging from 0.25 to 4 IC50 of inhibition of proliferation, over a 9 months period. Then, we characterized their resistance profile in term of ABC transporter and topoisomerase II expression.

**Results:** As key observation, the intense selection pressure led solely to a moderate shift of EC50 in proliferation assay, not mediated through MDR1 nor MRP1, but more likely attributable to a reduction of topoisomerase II expression levels. Furthermore, we observed that F14512 retained its antiproliferative activity on cell lines overexpressing MDR1: CEM selected with vinblastine and A-549 selected with vinflunine, and P388 selected with vinorelbine, with an enhanced impact on the later model.

**Conclusion:** We demonstrated that MDR1 does not play a crucial role in F14512 resistance. However, we are currently investigating the link between the MDR1 phenotype, polyamine import and F14512 accumulation.

**Estrogens and Alkylphenols Promote Proliferation of the Seminoma-like TCam-2 Cell Line Through ERα36-dependent Pathways**

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**Background:** Seminoma, originated from carcinoma in situ cells (CIS), is one of the main causes of cancer in young men. Postpubertal development of these testicular germ cell tumors suggests a hormone-sensitive way of CIS cell proliferation induction probably stimulated by lifelong exposure to endocrine disrupting agents. In a first step to understand the mechanisms underlying the deleterious effects of endocrine disrupting compounds on germ cells, we aimed to decipher the estrogen-dependent transduction pathways in TCam2 cells. Then, we began to assess the effects of a [4-tert-octyl + 4-nonylphenol] mix on testicular germ cell tumors in vitro and in vivo.

**Material and Methods:** In this study, we used the unique seminoma TCam-2 cell line which do not express the canonical ERα6 estrogen receptor but ERα36, a truncated isoform retaining the DNA-binding, partial dimerization and ligand-binding domains and a specific C-terminus 27aa sequence. Cells were exposed to either estradiol at concentrations in the range of those detected in food and drinking water. In vitro, we performed cell prolifereation assays, siRNA- or shRNA-directed knockdown, microarray directed gene targeting and signaling pathways identification after short term (1h) or mid-term (24h or 48h) treatment. We also addressed the question of TCam-2 derived tumor growth in xenografted Nude mice treated with the [4-tert-octyl + 4-nonylphenol] mix.

**Results:** We demonstrated in vitro that estradiol and the alkylphenol mix trigger TCam-2 cell proliferation through ERA36-dependent pathways. We established that estradiol can activate GPER/CAMP/PKA signaling pathway. Strikingly, that ERα36 is (i) necessary for cell proliferation (ii) a downstream target of estradiol-activated GPER/PKA/CREB pathway, (iii) required for estradiol-dependent EGF expression. The [4-tert-octyl + 4-nonylphenol] mix signaling pathway is clearly ERA36 dependent but seems to be partially non-estrogenic. Finally, we show that the [4-tert-octyl + 4-nonylphenol] mix stimulates tumor growth in TCam-2 xenografted Nude mice.

**Conclusions:** Our results highlight the functional role of ERA36 in context of seminoma cell proliferation and the importance of testing ERα36 in vivo as a possible marker for endocrine disruptor susceptibility.
slight elevation in $K_{\text{M_{R,BASE}}}$ value. The tertiary structure of WT and mutant PAD4 enzymes were examined by protein intrinsic fluorescence, demonstrating that some of the mutant enzymes displayed conformations different from that of WT. These conformational changes, however, did not bring about a considerable influence on subunit-subunit interactions, suggesting that mutation of these amino acid residues did not cause dissociation of PAD dimers into monomers. Here, we provide direct evidence for the need of Ca3 and Ca4, with these extra Ca2+ ions required for full activation of the enzyme.

**289** Characterization of a Mouse Model of Wound-induced Tumour Formation

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Mice that constitutively overexpress MEK1 specifically in the differentiated layers of the epidermis provide a mouse model of wound-induced tumour formation. The skin of these mice exhibits epidermal hyperproliferation and chronic inflammation [1]. When a full thickness wound is made in the back skin up to 80% of wounds form a benign tumour (piloma) within 30 days. Once formed, the tumours rarely regress and some progress to low-grade squamous cell carcinomas (keratoacanthomas). Previous work in the lab has established that tumour formation is dependent on recruitment of an inflammatory infiltrate, and that $\beta$-T-cells and macrophages are involved [2]. We are currently looking into the inflammatory mechanisms that are involved in tumour formation to be able to pinpoint crucial signalling pathways involved in tumour initiation.

Reference(s)


**290** Modifying TGF-beta Signalling and MEK Inhibition in Melanoma

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Introduction: Melanoma is an aggressive skin cancer that displays resistance to current chemotherapies. As over 90% of melanomas display constitutively active RAF/MEK/ERK signalling, a range of inhibitors targeting this pathway has been developed. The clinical effectiveness of these inhibitors is hampered by the presence and development of resistance. Moreover, MEK inhibitors seem to fail to induce cell death. Over-coming the resistance to cell death induced by MEK inhibitors is critically important to improve clinical outcomes. As the transcription factor MITF is known to be a crucial regulator of survival and proliferation in melanoma, we investigated its role in the resistance to MEK inhibitors.

Material and Method: MEK inhibitor PD184352 and recombinant TGF-beta were used in cell survival assays. Knock-down experiments were performed using siRNAs and shRNAs. A novel xenograft tumour growth model in zebrafish was utilised. A375 melanoma cells were treated with PD184352 for a pro-longed period of time in order to establish resistant cell lines.

Results and Discussion: Melanoma cell lines can be both intrinsically resistant to MEK inhibitors or be forced to acquire resistance when grown in the constant presence of the inhibitor. By double treating with TGF-beta and MEK inhibitor we can cause a significant increase in caspase-3 dependent apoptosis, in both intrinsically and acquired resistant melanoma. Furthermore we show that this co-operative effect is also produced by a beta and MEK inhibitor we can cause a significant increase in caspase-3 dependent apoptosis, in both intrinsically and acquired resistant melanoma.

Conclusion: We have shown that modifying TGF-beta signalling can sensitise melanoma cells to MEK inhibition. We therefore identify a promising approach for improving MEK inhibitor efficacy.

**291** Comparison of Conventional Cell Lines and Improved Models for Ovarian Carcinoma – Drug Targets and Cancer-stem Cell Markers

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Introduction: Ovarian cancer is the most lethal form of gynaecological cancers. Despite advances in therapy, only 30% of patients with advanced-stage ovarian cancer survive five years after diagnosis, exemplifying the need for improved therapeutic and diagnostic tools. Still the common model systems for drug screening and functional studies are long-term FCS-cultured ovarian cancer cell lines. The resulting cell lines frequently do not resemble the original malignancies and drug screens performed with those cell lines might yield hits of limited relevance for patient-tumours. Further, phenotypic heterogeneity is lost and with it the possible presence of putative ‘cancer stem cells’. This ‘CSC potential’ might play a role in drug-resistance, recurrence and metastasis. For ovarian cancer however, no CSC subpopulation has been conclusively described so far.

Material and Methods: Tumor samples from ovarian cancer patients were processed under serum-free culture conditions. Primary ovarian cancer cell lines were established from the first passage xenograft or directly from patient material. For in vivo experiments primary ovarian cancer cell lines were injected into immunocompromised mice. Established primary ovarian cancer cell lines were analysed by mass spectrometry and gene expression profiling to define putative subtypes for serious ovarian carcinoma.

Results and Discussion: We developed advanced in vitro and in vivo models for ovarian cancer, which show a high degree of genetic and histological identity with the original patient tumor. Unlike conventional FCS-based cultures this system retains the molecular heterogeneity of the human malignancy. Xenografts grown in immunocompromised mice were classified as serous ovarian carcinoma by histomorphology and expression of ovarian cancer specific markers CA125 and WT1. The in vivo models also recapitulate the patient disease by metastatic spread to liver and diaphragm and the development of ascites. Our advanced models facilitate screening for novel diagnostic and therapeutic targets in serious ovarian carcinoma, not accessible with previous cell lines.

Conclusion: In the past, model systems for ovarian cancer have been incapable of accurate mimicking the human disease. Here we present a model system, which combines CSC culture with xenotransplantation and models the human disease more faithfully. This model allows studying metastasis development and drug resistance in serious ovarian cancer, uncovering potential therapeutic targets previously not accessible.

**292** Morgana Haploinsufficiency Induces a Myeloproliferative Disorder Like-chronic Myeloid Leukemia

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Background: Morgana is an ubiquitously expressed protein that behaves like an HSP90 co-chaperone and protects cells from cell death induced by different stress stimuli. Mutations in morgana result in centrosome amplification and lethality in both drospila and mouse. In mice, morgana forms a complex with Hsp90 and Rho Kinase 1 (ROCK I) and Rho Kinase II (ROCK II), and directly binds ROCK, inhibiting its kinase activity and leading to centrosome amplification.

**morgana**−/− primary cells display an increased susceptibility to neoplastic transformation and **morgana** +/+ mice are more prone to tumor development after treatment with a chemical carcinogen.

Material and Methods: To characterize the role of morgana haploinsufficiency in tumor onset, we assessed the susceptibility of **morgana** +/+ mice to spontaneous tumor formation during their lifespan. In particular we evaluated mice for hematologic illness through observation, physical examination and blood counts at regular intervals. We characterized the hematologic disease by necropsy, tissue analysis, blood and bone marrow cells suspensions and histochemical stainings. In order to link mouse phenotype with human disease, we evaluated morgana transcript and protein expression in leukemia patient samples.

Results and Discussion: Here we show that about 40% of **morgana** +/+ mice get sick and die mostly between 12 and 16 months of age. Morgana haploinsufficiency in mice induces with age a fatal myeloproliferative disorder like-chronic myeloid leukemia (MDP like-CML) manifested by severe anemia, significant leukocytosis with neutrophilia in the peripheral blood along with myeloid hyperproliferation in the bone marrow and spleen and myeloid cell
A New Genetic Mouse Model to Identify the Role of the Immune Protein Kinase WNK2 Was Correlated With Poor Outcome and Prostate Cancer

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Material and Methods: Insertion of an 'intron-gene-trap' flanked with loxp motifs into the first exon of the Myd88 gene locus leads to global inactivation of Myd88 expression (Myd88-/-), faithfully phenotyping a global gene knock-out. Tissue-specific re-expression of Myd88 in mice is mediated based on the Cre-recombinase. Breeding of Myd88-/- mice with LysMCre or p21Ink-Cre mice leads to tissue-specific excision of the 'intron-gene-trap', retaining endogenous regulation of gene expression. Myd88 expression and successful reconstitution of TLR-signaling was detected in either myeloid cells (Myd88+/M) or intestinal epithelial cells (Myd88+/I). Subsequently, these animals were mated with established genetic mouse models for human colon cancer (p53-KrasG12PV12 and ApcMin/+). Results: Global Myd88 deficiency significantly decreased development of tumors, induced by either oncogenic KRAS or loss of function of the tumor suppressor Apc. Re-expression of Myd88 in intestinal epithelia and tumors partially restored tumor growth. Moreover, activation of the MAPK/cMyc pathway was independent of Myd88 expression in intestinal epithelia and tumors. On the other hand, reconstitution of Myd88 expression in myeloid cells triggered tumor development indistinguishable from parental ApcMin/+ mice. Conclusion: Our preliminary data indicate that Myd88-mediated signaling in myeloid cells, but not in epithelial-derived cancer cells, is crucial for intestinal tumor development.
Multidrug Resistance in Hepatocellular Carcinoma – Correlation With 18F-FDG Uptake (Preliminary Results)

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With 18F-FDG Uptake (Preliminary Results)

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Results and Discussion: We utilized published clinical expression array datasets and AR chromatin immunoprecipitation (ChIP) to identify functionally linked genes that are significantly overexpressed in localized prostate cancer and AR antagonist target genes. We found that a process sensing changes in metabolic flux, hexoseamine biosynthesis (HBP), is altered at the level of gene expression in prostate cancer.

Our bioinformatics approach suggested that HBP might be androgen responsive in prostate cancer cells. Therefore, we utilized two AR positive prostate cancer cell lines and observed a clear up-regulation of HBP genes upon androgen stimulation, which was followed by a robust increase in protein level. Downstream we observed effects on the expression, glycosylation and phosphorylation status of growth factor receptors.

Conclusion: Our work implies that metabolic changes in prostate cancer cells create a feedback loop which affects the signaling via growth factor receptors. This could generate a self-reinforcing signaling cascade and requires further investigation.

Hierarchical EMT-TFs Regulation Controls Epithelial Mesenchymal Transition

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Background: Epithelial mesenchymal transition (EMT) is an essential process in normal development as well as cancer progression. EMT hallmark traits are the loss of E-cadherin, the induction of mesenchymal markers and the acquisition of a motile/invasive phenotype. Cancer cells that activate EMT acquire more aggressive phenotype facilitating tumor spreading and facilitating metastasis. Several EMT-TFs have been reported: SNAIL family members (SNAIL1 and SNAIL2), bHLH family (E47 and TWIST) and ZEB proteins. Nevertheless, the hierarchical regulation among EMT-TFs is largely unknown. Recently, miR200 family has emerged as a key regulator of EMT and a negative feedback loop between ZEB and miR200 family has been reported. Methylation of the miR200 family by hypermethylation or in tumor suppressor gene has been recently reported. In the present study, we have analyzed the functional interrelation between different EMT-TFs and miR200s.

Materials and Methods: MDCK cell line was used as an EMT-induced model. Overexpression of different EMT-TFs (Snail1/2, Zeb1/2, Twist1/2, E47) or some regulators (Id1, LOXL2) was achieved by stable transfection. Knock down assays were performed by lentiviral infection of siRNAs in selected EMT cell models. Western blot, immunofluorescence, microarray analysis, qPCR, and MassARRAY platform were used to analyze protein, mRNA or methylation status in the different models.

Results and Discussion: As previously reported for the Snail and E47 factors, EMT-induced cells by the various EMT-TFs shared similar invasive phenotype, lack E-cadherin expression and upregulate N-cadherin and other EMT markers. Moreover, EMT-induced cells showed reduced proliferation and increased basal apoptosis suggesting that the different EMT-TFs shared similar EMT triggering pathways. Comparative gene profiling analyses support these findings but also indicate differential targets of the various EMT-TFs. Interestingly, knock-down assays in EMT-induced cells indicate the participation of distinct EMT-TFs in different mechanisms of EMT regulation: initiation and maintenance. Thus, knock-down of some EMT-TFs induces a complete mesenchymal-epithelial transition (MET), while knockdown of others abolished the EMT inducing ability of Snail factors, indicating their participation in EMT initiation and the hierarchical interrelation of different EMT-TFs. Finally, we are investigating the expression levels of the miR200 family and its methylation status in all the EMT models in order to identify feedback regulatory loops between both essential EMT regulators.

Conclusions: Control of the EMT process is achieved by EMT-TFs and miR200 regulation. Similar processes were triggered after EMT induction in MDCK cells by distinct EMT-TFs. We have identified two subgroups of EMT-TFs: inducers and keepers of the EMT confirming the plasticity of this essential process.

A Role for BRCA1 in the Regulation of Ribosome Biogenesis

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Introduction: BRCA1 is a well characterised tumour suppressor gene expressed in all mammalian cells. It is found to have many roles including cell cycle regulation, DNA damage repair and in transcription. Disruptions in these processes are thought to lead to an increased rate of ribosomal biogenesis, the process enabling cell growth and proliferation via the synthesis and processing of the rRNA.

Materials and Methods: Immuno-fluorescence images were taken using confocal microscopy to identify any co-localisation of A135 (Pol I) antibody with BRCA1 in breast, colon and prostate cancer cell lines. Immunoprecipitation using BRCA1 antibody was performed on different cell lines. Reverse transcription-quantitative PCR and Sequenom MassArray® MALDI-TOF platform were used to analyze protein, mRNA or methylation status in the different models.

Results and Discussion: Immuno-fluorescence images suggested possible co-localisation of BRCA1 with factors involved in Pol I transcription i.e. A135 (Pol I) UBF, TAF63 and TBP (SL1). BRCA1 co-immunoprecipitation studies in the same cell lines showed these factors are pulled down along with BRCA1. ChIP assay on breast cancer cell lines revealed that BRCA1 is present at the promoter and throughout the rDNA repeat. When UV induced DNA damage occurred a significant decrease in the level of BRCA1 in the rDNA repeat, however when DNA damage was induced with etoposide the level decreased dramatically. Preliminary data carried out in a breast cancer cell line (T47D) shows a decrease in the ratio of rDNA copy number in the larger subunit to the smaller subunits when BRCA1 is knocked out or the overall level reduced.

Conclusion: Results suggest that BRCA1 can potentially directly interact with various components of Pol-I transcription machinery and may have a possible regulatory role as it is present at the promoter of the rDNA repeat and levels are shown to increase with UV induced DNA damage. Its role in ribosomal biogenesis appears significant as when absent the levels of processing decrease.

Materials and Methods: LNCaP, VCaP and RWPE-1 cell lines were maintained according to ATCC guidelines. Cells were treated with synthetic androgen receptor (AR) agonist or antagonist. Expression levels were assessed using RT-PCR and Western blotting. Glycosylation was assessed by lectins to identify modified proteins. Phosphorylation was explored using a commercial phospho-specific antibody array.

Results and Discussion: Therefore, we utilized two AR positive prostate cancer cell lines and observed a clear up-regulation of HBP genes upon androgen stimulation, which was followed by a robust increase in protein level. Downstream we observed effects on the expression, glycosylation and phosphorylation status of growth factor receptors.

Conclusion: Our work implies that metabolic changes in prostate cancer cells create a feedback loop which affects the signaling via growth factor receptors. This could generate a self-reinforcing signaling cascade and requires further investigation.
Interleukin-6 Expression and JAK-STAT Signalling in Prostate Cancer Stem Cells

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Introduction: Prostate cancer is the most common cancer in men in the UK and is a major health problem worldwide. Currently there is no effective therapy for the recurring form of prostate cancer. Treatment failure may be due to the existence of cancer stem cells (CSCs). Gene expression profiling of prostate cancer stem cells (CD44+/CD133+) showed that IL-6 and members of the JAK-STAT pathway were over-represented in CSCs relative to differentiated cells. If the JAK-STAT signalling pathway is engaged in the maintenance of stem cells for prostate cancer, this could represent a potential therapeutic target.

Materials and Methods: The mRNA and protein expression of IL-6 in primary prostate cancer and benign cell cultures were analysed by qRT-PCR and ELISA. Immunofluorescence was performed to determine IL-6 receptor localization and Western blots were used to determine levels of phosphorylated STAT3. In vivo studies were carried out on a panel of ‘near patient’ xenografts of prostate cancer in Rag2−/−; C−/− mice, to determine the effect of inhibiting STAT3 directly on prostate cancer (stem) cell fate.

Results and Discussion: IL-6 mRNA and protein were highly expressed in the CSC population compared to progenitor cells (CD133+/CD44hi) of primary prostate cancer cells. Significantly, this expression pattern was apparent in samples originating from patients with high Gleason grade tumours, but not from patients on hormone treatment or those with benign disease. The IL6 receptor was also expressed and STAT3 phosphorylated in both primary prostate cancer and benign samples indicating constitutive activation of the pathway. Inhibition of phosphorylated STAT3, in vivo, resulted in inhibition of tumour growth. In addition, a higher proportion of CD24+ luminal cells was observed.

Conclusion: Prostate CSCs express high levels of IL-6 and the associated IL-6 receptor suggesting that these cells are dependent on the JAK-STAT signalling pathway via an autocrine feedback loop. Supporting evidence for this is that STAT3 is constitutively active in primary prostate cancer cells. Preliminary in vivo data confirms this hypothesis, as treatment with a pSTAT3 inhibitor resulted in tumour growth inhibition. Initial results suggest that the basal tumour cells (CD44hi) differentiate into a more luminal phenotype (CD24+) following inhibition of IL-6 suggesting that the progenitor cells are dependent on the JAK-STAT signalling pathway to maintain an undefined differentiated phenotype.

Transcriptional Cooperation Between p53 and Estrogen Receptors in a Breast Cancer Model

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Background: Previous reports have revealed a complex crosstalk between p53 and estrogen receptors (ERs) including transcriptional cooperation mediated by non-canonical cis-elements. The tumor suppressor p53 is a sequence-specific transcription factor activated by many stress signals that modulates genes involved mainly in apoptosis or cell cycle control. ERs are steroid hormone receptors and their primary response to estrogens is modulation of gene expression. p53 and ERs are poorly responsive to p53 alone. Hence, the cooperation of p53 and ERs expands the p53 transcriptional network with implications for the cancer stem cells with important impact for prostate cancer maintenance, cell adhesion, development or differentiation (such as TLR5, KRT15, CD26, NOTCH1, GDNF, INPP5D). The biological consequences of the identified p53-ER-dependent gene expression co-regulation are under investigation.

Conclusions: p53 and ERs modulate distinct cellular responses but our results indicate a synergistic cis-element-based transcriptional cooperation that can also occur and appears to be related to non-canonical REs that are not or poorly responsive at all to p53 alone. Hence, the cooperation with ERs expands the p53 transcriptional network with implications for the appearance and possibly the treatments of cancer.

Mammaglobin Expression and Receptor Status in Primary and Recurrent Breast Cancer

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Background: Human mammaglobin has been reported to be exclusively expressed in mammary epithelium and over expressed in some breast cancers. The mammaglobin protein is dramatically increased in proliferating breast cells and its production ceases upon breast epithelial cell differentiation. Therefore, mammaglobin synthesis may be involved in breast cell proliferation which would correlate with the over expression seen in some breast cancers.

Materials and Methods: 183 breast specimens were analysed by immunohistochemistry for mammaglobin A expression; 17 non-cancerous breast tissue, 143 were primary breast cancers and 23 were recurrent breast cancers. Stained sections were screened under the microscope with sections regarded as positive when >10% of lesional cells stained positive. For comparison purposes histological grade, tumour type, tumour size, ER, PR, Her-2 status and presence/absence of nodal metastasis were recorded. Correlation was performed for breast conditions. The study had ethics approval.

Results: Positive mammaglobin expression was observed in 52% breast samples studied (52% primary and 48% recurrent). Positive expression was associated with benign or low grade tumours with 59% benign, 67% grade 1, 53% grade 2 and 44% grade 3 breast tumours demonstrating positive expression. Of the 21 patients with recurrent breast cancer, the same mammaglobin expression was observed in only 43% paired primary and recurrent tumour samples. Two of these patients had a second tumour recurrence but both of these samples had negative mammaglobin expression.

Mammaglobin expression in primary tumours positively correlated with both ER status (58% correlation; p<0.05 Chi-squared) and PR status (81% correlation). There was no correlation with lymphatic invasion, tumour size or HER-2 status.

Conclusions: Positive mammaglobin protein expression was found in a greater proportion of benign and low grade breast tumours than higher grade. The expression in primary tumours was significantly associated with both ER and PR status. Since positive ER status and lower tumour grade are linked with a better prognosis for breast cancer patients, then mammaglobin A protein expression may also be associated with better prognosis.

Spontaneous Hybrids Between Breast Cancer and Multipotent Stromal Cells Acquire a Mixed Gene Expression Profile While Maintaining the Histopathological Appearance of Breast Carcinoma

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Introduction: In the course of breast cancer progression, cell sub-populations exhibiting cytogenetic abnormalities along with invasive/pro-metastatic features are generated. Some of these cells break free from the primary tumor, invade the microvasculature, travel and establish foci at distant sites. Sequential genetic mutations, epithelial-mesenchymal transition, interaction with local stroma as well as formation of hybrids between cancer cells and normal bone marrow-derived cells have been advocated as tumor progression mechanisms. We report here the spontaneous in vitro formation of heterotypic hybrids between human bone marrow-derived multipotent stromal cells (MSC) and two different breast carcinoma cell lines.

Materials and Methods: We co-cultured human MDA-MB-231 (MDA) or MA11 breast carcinoma cells with MSC under conditions favoring the selection of hybrids. Hybrids were characterized in vitro, and implanted into immune-deficient mice. The histopathology, expression profile and coding SNPs of the xenografts were investigated.

Results and Discussion: Hybrids showed a predominant mesenchymal morphology, mixed gene expression profiles and increased DNA ploidy. Both MA11 and MDA hybrids were tumorigenic in immune-deficient mice, and some MDA hybrids had increased metastatic capacity. Both in culture and as xenografts, hybrids underwent DNA ploidy reduction and morphological reversal to breast carcinoma-like morphology, while maintaining a mixed breast
cancer-mesenchymal expression profile. Both parental partners contributed genes to hybrid tumors and metastasis. Since MSC migrate and localize to breast carcinoma, our findings indicate that formation of MSC-breast cancer cell hybrids is a potential mechanism of generation of invasive/metastatic breast cancer cells.

Conclusion: Our findings reconcile the fusion theory of cancer progression with the common observation that breast cancer metastases are histopathologically similar to the primary neoplasm. Novel anti-breast cancer strategies may derive from prevention of hybrid formation and/or selective targeting of hybrids.

105 Identifying the Mechanisms of Cross-talk Between Breast Tumor Cells and the Bone Microenvironment for the Targeted Treatment of Bone Metastases
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Background: Metastatic dissemination is a major cause of morbidity and mortality associated with malignant breast carcinoma. The bone is a major site of tumor metastasis, and provides a reservoir for metastatic cells that can enter into a quiescent state and remain latent for decades after dissemination. The unique cross-talk between tumor cells and stroma of metastatic sites that favor tumor survival is central to the pathophysiology of metastases. Improved understanding of these cross-talk mechanisms promises to uncover therapeutic opportunities that focus on both the tumor and the microenvironment to prevent and treat bone metastases.

Materials and Method: This project examines the gene expression profiles of defined populations of bone stroma to identify transcriptional changes that occur in response to tumor metastases. We have taken advantage of both xenograft and allograft models of breast carcinoma, which have a high propensity for bone metastases following intracardiac injection in immunodeficient and immunocompetent mice respectively. Bioluminescence imaging tracks dissemination and cell sorting isolated defined cell populations from the bone environment. Excluding cells of the hematopoietic and erythroid lineages (CD45+TR119-), we isolated; endothelial cells (CD31+), osteoblasts (Sca1+CD31+CD61+) and mesenchymal progenitors (CD31+Sca1+CD61+) from native and tumor-bearing mice and compared the bone stroma populations by a genome wide-transcriptome study. To investigate the effect of bone microenvironment on disseminated tumor cells, we also compared the expression profile of primary tumors isolated from the mammary fat pad with metastatic tumor cells from either the bone or lung.

Results: Microarray analysis revealed that the bone stroma is significantly affected by breast carcinoma dissemination. Multiple components of key molecular pathways involved in tumor development (TGFβR, PDGFβR, EGFR, HGF and IGFR signaling pathways) are modulated in the stroma of tumor-bearing mice. PI3KCa, a downstream effector of several of the aforementioned pathways, is also up-regulated. We also uncovered transcripts for many surface associated proteins whose expression is reduced in cancer cells that occur in response to tumor metastases. We have hijacked the bone, compared to tumor cells growing in the mammary fat pad. Based on these findings, the effects of PI3K-mTOR inhibition have been confirmed in an in vitro approach for the discovery of marker sets for patient stratification according to subtype has been established. Furthermore, we used gene expression data to predict subtype-specific pathway dependencies and drug vulnerabilities. Those predictions were confirmed in an in vitro drug screen. In particular we found that the three subtypes differ widely in their drug sensitivity profiles, demonstrating the need for patient stratification in PDAC. With our model system we provide the means to discover such subtype-specific drugs.

Conclusion: Our culture model preserves all three PDAC subtypes, providing the unique opportunity to characterize the complex environment of this cancer. The observed differences in drug sensitivity underscore the need for stratification of patients with pancreatic cancer. With the demonstration of subtype-specific markers and development of a pre-clinical model for drug testing, we provide the tools to develop improved therapies for this deadly disease.

207 Growth Inhibition Effects of Human Breast Cancer Cell Line by Ursolic Acid
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Background: Ursolic acid (3-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid derived from leaves, berries, fruits, and flowers of medicinal plants, such as Rosemarinus officinalis. Ursolic acid has been shown to inhibit tumor growth, tumor promotion, and suppress angiogenesis.

Materials and Methods: The growth inhibition of ursolic acid in MDA-MB-231 cells was done through by MTT assay and microscopy. The molecular mechanism pathways were examined by FACS, western blotting.

Results: Ursolic acid induced apoptosis through the extrinsic pathway, including the activation of caspase-3, and degradation of PARP. Furthermore, ursolic acid induced cell cycle arrest in the G0/G1 phase and increased sub G1. In addition, ursolic acid inhibits the PI3K/Akt signaling pathway.

Conclusions: These data indicate that ursolic acid induces apoptosis in MDA-MB-231 cells. Our data clearly indicate that ursolic acid could be used as a potential anticancer drug for breast cancer.

208 Comparison of Deuterium Oxide Dilution Technique With Bioelectric Impedance Analysis and Anthropometry for Assessment of Body Composition in Cancer Children
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Background: Bioelectric impedance (BIA) and anthropometric analysis are practical, non-invasive, safe and portable methods for assessing body composition. The gold standard for measurement of body composition is isotope dilution technique. We aimed to determine the body composition of children with cancer using all these methods before and after chemotherapy.

Material and Methods: Fourteen children (aged 5.6 to 13.6 years old) were enrolled and classified as having hematologic or solid tumors. They had body composition measured by deuterium oxide dilution technique, bioelectric impedance analysis, and anthropometric data before the first chemotherapy and after 3 and 6 months of therapy.

Results and Discussion: The patients in hematologic tumor group improved their weight, stature, body mass index, hip, waist and arm circumferences, subcapsular skinfold thickness, as well as had an increase in fat-mass with isotopic dilution technique during the chemotherapy treatment. In the solid tumor group the children showed a reduction in fat-free-mass when assessed by BIA. There was a positive correlation between triceps skinfold thickness and fat-mass determined by BIA and by deuterium oxide dilution technique. The arm muscle circumference correlated with the fat-free-mass estimated by BIA and by deuterium oxide technique. There were no differences in energy and macronutrients intake at the three moments of the research.

Conclusions: Patients with hematologic tumors had an increase in body weight, stature and fat-mass, which was not observed in solid tumors. The good correlation between anthropometry and deuterium oxide dilution technique and BIA shows its applicability in clinical practice.
Introduction: One of the mechanisms governing protein denitrosylation is the system thioredoxin/thioredoxin reductase (Trx/Trxr). Alteration of this enzymatic system may impair S-nitrosothiol (SNO) homeostasis in tumor cells, providing new insights into the role of nitric oxide in cancer and its role in tumor progression and antitumor treatment.

Material and Method: MCF-7, MDA-MB-231 and BT-474 cells were pretreated or not with the highly specific TrxR inhibitor auronafin and exposed to different doses of S-nitroso-L-Cysteine (CSNO). S-nitrosylated proteins were detected using the ‘biotin-switch’ assay. Subcellular localization of ER alpha was analyzed by confocal microscopy. Oncomine database was explored for TrxR (TXNRD1) expression in ER- and ER+ breast tumors.

Results: In all the three cell lines, a high CSNO dose (500 μM) reduced cell proliferation and cell cycle arrest in G0/G1 phase induced by pretreatment with auronafin. Augmented levels of S-nitrosylated proteins were observed in MCF-7 cells treated with 500 μM CSNO, and this effect also was potentiated by pretreatment with auronafin. However, treatment with auronafin and 100 nM CSNO enhanced cell proliferation of estrogen receptor positive (ER+) MCF-7 cells, but not of MDA-MB-231 (ER−, mut p53), or BT-474 (ER−, mut p53) cells. This cell growth was associated with Akt and Erk 1/2 phosphorylation, augmented S-nitrosylation expression and was abrogated by the ER antagonist fulvestrant or the p53 specific inhibitor pifithrin-α. The specific silencing of ERα-alpha expression in MCF-7 cells also abrogated the growth effect of TrxR inhibition. Erogstenic deprivation potentiated the pro-proliferative effect of SNO homeostasis impairment in MCF-7 cells. Moreover, the subcellular distribution of ER-alpha was altered, with a predominantly nuclear localization in those cells with impaired SNO homeostasis. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression. When Oncomine database was interrogated, positive ER status in breast tumors was found to be associated with significantly lower levels of TXNDR1 expression.

Conclusion: The function of LOX as a transformation-suppressing gene in human and rat fibroblasts was confirmed by overexpression in HRAS-transformed cells. LOX overexpression in BJELR cells, a HRAS-transformed derivative of BJELB, promoted the formation of reactive oxygen species (ROS) in physiological conditions. LOX overexpression in BJELR cells reversed the transformed morphology, inhibited proliferation by 30%, and abrogated anchorage independent growth by more than 90%. Ectopic stable LOX expression controlled by an ubiquitin promoter induced cell cycle arrest in G0/G1 in BJELR cells. In RAS-transformed rat fibroblasts, LOX expression also significantly inhibited focus formation.
LARP1 Regulates the Site-specific Synthesis of Proteins Required for Cancer Cell Invasion and Migration

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Introduction: LARP1 is a member of the LA-related protein (LARP) family, which shares a LAM domain (LAM) and RRM domain with genuine LA proteins. LARP family members play a role in mRNA translation and RNA homeostasis. We have previously demonstrated that LARP1 enhances protein synthesis by binding poly-ADP-ribose binding protein (PARP) and promoting PARP degradation. After its ectopic expression, LARP1 localises to lamellipodia at the cellular leading edge and interacts with cytoskeletal proteins: actin, tubulin, and the motor proteins non muscle Myosin IIA-IIB and VI. In light of these findings, we hypothesise that LARP1 activates cancer cell migration and invasion by promoting the site-specific synthesis of key migratory proteins at the leading edge.

Materials and Methods: HeLa cells were treated with either the pro-migratory factors bFGF or TGF-β1, or the Myosin light chain kinase inhibitor (MLCK) before performing protein extraction and western blot analysis or immunofluorescence staining. HeLa cells were transiently transfected with Flag-LARP1 (empty vector construct for 48 hours before performing a wound healing assay. SKOV3 cells were transfected with PTX-LARP1 or PTREX vector. Stably transfected clones were selected and used in a MatrigelTM coated trans-well assay.

Results and Discussion: In HeLa cells, LARP1 expression was increased following treatment with the growth factors TGF-β and bFGF. Confocal microscopy of these stimulated cells demonstrated an increased number of pseudopodia as well as relocalisation of LARP1, PARP, and eIF4E to the leading edge. An invasion assay, using a SKOV3 derived stable cell line over-expressing LARP1 in MatrigelTM coated trans-well, showed a 50% increase in number of invading cells compared to controls. An increased rate of migration was also observed in HeLa cells in which LARP1 was ectopically over-expressed. Again, confocal imaging showed co-localisation of PARP, eIF4E, and LARP1 at the leading edge of these migrating cells. These proteins were confirmed to be in complex by co-immunoprecipitation. Localisation of LARP1 eIF4E and PARP at the leading edge was abrogated following treatment with MLCK suggesting it is a myosin-dependent effect.

We have shown that over-expression of LARP1 increases both migration and invasion in two cancer cell lines and that LARP1 is up-regulated following stimulation with TGF-β and bFGF. LARP1 co-localises and is in complex with the translation initiation proteins eIF4E and PKB at the leading edge suggesting that localised protein synthesis is occurring here and this is dependent on myosin motor protein.

Conclusions: We conclude that, by activating protein synthesis at the cellular leading edge, LARP1 is required for cancer cell migration and invasion.

Cyclic AMP Inhibits the Malignant Phenotype of Human Colon Cancer Cells Via an Epac-dependent Mechanism

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Introduction: Colorectal cancer (CRC) is the third most common neoplasm and a major contributor to cancer-related deaths in the western world. Although the therapeutic strategies developed for the treatment of CRC have progressively improved over the last decade, the 5-year survival rate of CRC remains poor, with the 5-year survival rates being less than 40% in most series. Therefore, there is an increasing need for developing novel and more effective therapies for this disease. Many phenotypes of cancer cells are regulated by cyclic AMP (cAMP). However, very little is known about the role of cAMP in CRC.

Material and Methods: Human CRC cells (HCT116) were used. forskolin was used to elevate intracellular cAMP levels. 8-bromo-cAMP was used to selectively activate protein synthesis directly activated by cAMP (Epac). Viability and apoptosis were measured via luminescent assays for ATP count and Caspase 3/7 activity, respectively. mRNA levels were measured by RT-PCR.

Results: Our results show that forskolin, a cAMP-elevating agent, inhibited proliferation of CRC cells in concentration (0–10 μM) and time (0–96 hours) dependent fashions. This effect of cAMP was mimicked by the selective Epac-agonist 8-β-(4-chlorophenylthio)-2′-O-methyladenosine-3′,5′-cyclic monophosphate (8CPT-2Me-cAMP) (0–150 μM) and by the PKA-selective activator, N6-benzoyl-cAMP (100–1000 μM). Inhibition of PKA by H89 (2 μM) significantly diminished the cAMP-elicited effect. Similarly, expression of an dominant negative mutant of Epac abolished the cAMP elicited effect. Concomitantly, activation of either PKA or Epac induces apoptosis, marked by increased Caspase 3/7 activity. Our results also show that Epac regulates cell-cell adhesion which is critical for CRC invasion and malignancy. Indeed, Epac increased the mRNA expression of both E-cadherin and occludin. Importantly, Epac activation also appears to potentiate the functional integrity of tight junctions in these cells, evident by its ability to increase transepithelial resistance. Conclusion: cAMP, Epac, acting via Epac1 and Epac2, reduces the malignant phenotype of human CRC cells by inducing growth arrest, apoptosis and cell-cell adhesion. These results offer an unprecedented look into the role of cAMP in CRC, especially that mediated by Epac. A better understanding of the molecular mechanisms is critical for the development of new therapeutic strategies.

Serum Immune Marker Profile in Colorectal Carcinoma

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Background: Colorectal cancer (CRC) is one of the three most common cancers worldwide. The prognosis of CRC is influenced by a complex interplay between the tumor and the host inflammatory response. Cytokines are key regulators of immune response and can modulate tumor growth and microenvironment by mediating interactions between cancer cells and infiltrating inflammatory cells. The pathogenesis of CRC is accompanied by alterations in cytokine production; local cytokine environment is thought to polarize from T helper (Th) 1 into Th2 type of cytokines along the T cell receptor (TCR) αβ effector lineage. A panel of cytokines is characterized by a pro-inflammatory Th1 profile and a regulatory (Th2) profile.

Methods: The preoperative serum levels of 27 cytokines were measured from a series of 148 CRC patients and 86 healthy controls using Bio-Plex Pro Human 27-Plex Cytokine Panel (Bio-Rad, Hercules, CA, USA). The pathogenesis of CRC is associated with the serum concentration levels were assessed with univariate analyses and clinicopathological association of serum cytokine pattern in CRC.

Results: The five most predictive cytokines included in the logistic regression model was generated to assess the mutual relationships of the differences observed and to evaluate the value of serum cytokine profile in discriminating the CRC patients from the controls.

Conclusions: The results of serum levels of several cytokines were significantly altered: CRC patients had higher serum levels of platelet derived growth factor subtype BB (PDGF-BB), interleukin (IL)-6, IL-7, and IL-8 compared to healthy controls, whereas levels of monocyte chemotactic protein-1 (MCP-1) were lower in CRC. The most predictive cytokines included in the logistic regression model were high IL-8, high IL-6, low MCP-1, low IL-1 receptor antagonist (IL-1ra), and low 10 kDa interferon-gamma-induced protein (IP-10). Receiver operating characteristics (ROC) analysis for the model yielded an area under the curve (AUC) of 0.890 in separating patients with CRC from controls compared to AUC of any of the cytokines alone. Increased levels of IL-8, IL-1ra, and IL-6 had the strongest association with high-stage disease. A metastasised disease was accompanied by an orientation towards Th2 cytokine milieu.
Conclusions: CRC is characterized by extensive and characteristic changes in serum cytokine levels. This study highlights the importance of studying serum cytokines as a group to observe relative expression level variations. Serum cytokine profile is a promising new biomarker for CRC, but the clinical value of this profile is yet to be confirmed.

Control of Breast Cancer Growth and Initiation by the Stem Cell-associated Transcription Factor TCF3

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Materials and Methods: 

Results: We found that TCF3 function is critical for breast cancer cell proliferation and tumor formation, and that in its absence tumor initiation capacity is dramatically reduced. Consistent with this, TCF3 overexpression confers stem cell-associated traits on breast cancer cells. We found that TCF3 controls gene expression programs associated with epithelial identity, thereby affecting cancer cell differentiation state. This function is conserved in the normal mouse mammary gland. In contrast to ES cells, in which TCF3 is triggered by mutations in Ecad has come from the finding that germline mutations in its gene (CDH1) are the only known genetic cause of Hereditary Diffuse Gastric Cancer (HDGC). We have previously shown that cancer-associated Ecad missense mutations frequently lead to destabilization due to folding defects, resulting in increased degradation, but the molecular mechanisms involved are not clear. Using a genetic functional screen in the Drosophila eye, we found that Drosophila DNAJ-1 interacts with WT and mutant human Ecad, suggesting that its human homolog DNAJB4 is a new Ecad regulator.

DnaJB4 Differentially Regulates Wt and Mutant E-cadherin in Cancer


Background: E-cadherin (Ecad) is a well known Tumor Suppressor Gene (TSG), with a clear invasion suppressor role, and its loss of expression is a hallmark of invasive carcinomas. The demonstration that tumorigenesis is strongly reduced in almost all lung tumors with EGFR activating mutations, compared to tumors with EGFR L858R mutation, indicates that EGFR are generally referred to as activating mutations as they seem to result in the increased activity of the receptor, leading to hyperactivation of downstream pro-survival pathways. The ARF tumor suppressor is at the crossroad of oncogenic and genotoxic pathways and plays a strong protective role against lung cancer development. Baseline data showing that ARF expression is strongly reduced in almost all lung tumors with EGFR activating mutations, we postulated that ARF is part of a failsafe mechanism protecting cells against untimely or excessive mitotic signals induced by EGFR signaling. Method: Knockdown of E-CAD in lung adenocarcinoma cells followed by overexpression of WT ARF or abolished (siRNA) expression vectors or abolished (siRNA) in H325S and H1975 cell lines derived from lung adenocarcinoma that carry the missense activating mutation L858R of EGFR and express low level of p44/42. Cell growth and apoptosis were studied after methylene blue and active caspase 3 staining respectively. Specific siRNAs and pharmacological inhibitors were used to characterize the signaling pathway controlled by ARF. siRNA against EGFR L858R or EGFR treatment were used in H1975/S325S and H1719 (wild type EGFR) cells respectively to study the role of activated EGFR in the p44/42 expression. Results: We demonstrate that p14ARF promotes apoptosis in lung tumor cells harboring the EGFR L858R mutation through the accumulation of phosphorylated STAT3 on Tyr705 residue, which leads to Bcl-2 downregulation. Using siRNA against PTP-RT, the phosphatase that specifically targets Tyr705 residue, we show that accumulation of pSTAT3-Tyr705 promotes EGFR L858R mutant cell death through Bcl-2 inhibition, thereby confirming the existence of a STAT3-dependent pro-apoptotic signaling pathway in these cells. Activation of EGFR either through mutation or after ligand binding represses p14ARF expression, and inhibits by this way the pro-apoptotic STAT3/Bcl-2 pathway in EGFR L858R mutant cells.

Control of Pancreatic Homeostasis by the E2F-p53 Regulatory Axis


Background: Cytokine profile is a promising biomarker for CRC, but the clinical value of this profile is yet to be confirmed. TCF3 promotes the expression of a subset of important Wnt signaling components, thereby affecting cancer cell differentiation state. This function is conserved in the normal mouse mammary gland. In contrast to ES cells, in which TCF3 expression is specifically high in aggressive tumors, most of the basal-like subtype. TCF3 encodes a transcription factor that plays critical roles in the regulation of embryonic and skin stem cells. We thus hypothesized that TCF3 may be involved in dictating the differentiation and tumorigenic traits of breast cancer cells.

Results: We found that TCF3 function is critical for breast cancer cell proliferation and tumor formation, and that in its absence tumor initiation capacity is dramatically reduced. Consistent with this, TCF3 overexpression confers stem cell-associated traits on breast cancer cells. We found that TCF3 controls gene expression programs associated with epithelial identity, thereby affecting cancer cell differentiation state. This function is conserved in the normal mouse mammary gland. In contrast to ES cells, in which TCF3 expression is specifically high in aggressive tumors, mostly of the basal-like subtype. TCF3 encodes a transcription factor that plays critical roles in the regulation of embryonic and skin stem cells. We thus hypothesized that TCF3 might be involved in dictating the differentiation and tumorigenic traits of breast cancer cells.

Conclusions: Our findings reveal that TCF3 plays crucial functions in controlling the proliferative capacity and epithelial identity of breast cancer cells, and present TCF3 as a novel link between stem cells and cancer.
Conclusions: These results identify a novel link between the p14ARF and EGFR pathways and suggest that mutant EGFR L858R counteracts the pro-apoptotic function of p14ARF by downregulating its expression to promote carcinomaogenesis.

[323] The VEGF-a Splice Variants VEGF165 and VEGF165b Differentially Control the Proliferation of Non Small Cell Lung Carcinoma Cells

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Background: VEGF-A is an endothelial mitogen and survival factor that plays a crucial role as a regulator of angiogenesis in healthy and neoplastic tissues. Multiple isoforms of VEGF-A are generated by alternative splicing. In 2002, a new family of VEGF isoforms termed VEGF165b, which results from the selection of a distal splice site in exon 8 of VEGF-A pre-mRNA, has been identified. VEGF165 and VEGF165b are the main expressed isoforms of both families. Despite a 94–98% homology with VEGF165, isoform, further studies have shown that VEGF165b acts as an anti-angiogenic protein. Besides endothelial cells, VEGF isoforms are also thought to hit tumor cells that express VEGF receptors including Non Small Cell Lung Carcinoma (NSCLC). To date, the function of this VEGF autocrine loop remains poorly understood. The purpose of this study was to investigate the effects of VEGF165 and VEGF165b in this setting.

Material and Method: H358 and H1299 human lung adenocarcinoma cell lines were transiently or stably transfected with a plasmid encoding VEGF165 or VEGF165b or treated with rhVEGF165 or rhVEGF165b ligand (R&D Systems). Activation of phospho-VEGFR1/VEGFR2 was detected by ELISA assays (R&D Systems) and western blotting using specific phospho-antibodies. VEGF165 and VEGF165b proteins were analyzed by immunohistochemistry in a series of 80 NSCLC including 43 adenocarcinoma and 37 squamous lung carcinoma.

Results: We showed that cells overexpressing VEGF165b proliferate and migrate faster than control cells. Activation of phospho-p38 MAPK and PI3K/AKT signaling pathways was detected in tumor cells treated with VEGF165b and did not correlate with VEGFR1/VEGFR2 activation. We further showed that tumor cells overexpressing VEGF165b are more resistant to apoptosis triggered by the VEGF tyrosine kinase inhibitor SU5416 as well as by platinum salts. Strikingly, treating cells with VEGF165b actually led to phospho-VEGFR1 accumulation and apoptosis. Anti-angiogenic therapies (VEGFR tyrosine kinase inhibitor and anti-VEGF antibody) as well as platinum salts increased the ratio between pan-VEGF and VEGF165b isoforms in favor of VEGF165b in tumor cells. Finally, we found that VEGF165 and VEGF165b splice isoforms are differentially expressed in situ in a series of 80 NSCLC.

Conclusions: These results are the first evidence of a distinct role of VEGF165 and VEGF165b splice variants in the control of proliferation/apoptosis in NSCLC and suggest that the VEGF165/VEGF165b ratio is involved in NSCLC response/escape to anti-angiogenic therapies and chemotherapies.

[324] Role of Lipid Bodies on Cell Cycle Progression

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Background: Lipid bodies (also known as lipid droplets) are organelles involved in lipid turnover, membrane traffic and intracellular signaling. Lipogenesis has been associated with poor prognosis in several neoplastic diseases, suggesting a role for these organelles in cancer development. We have previously reported that lipid bodies are centrally involved in the regulation of cell cycle progression.

Materials and Methods: NIH3T3 cells were synchronized through combination of confluence and serum starvation, and progression through cell cycle was assayed before or after 12, 24, 36 or 48 hours of serum supplementation by propidium iodide staining, by western blot analysis of cell cycle proteins, and by qPCR to assess cyclins mRNA expression. Lipid bodies regulation was assayed before or after 12, 24, 36 or 48 hour of serum supplementation, along with histone H3 phosphorylation after 48 hours. mRNA expression analysis of cyclins D2, E2, A2, and B2 confirmed that synchronized NIH3T3 cells progressed uniformly through cell cycle after serum supplementation. Using this model, we observed that cells arrested on G2/M phase showed a lower number and presence of lipid bodies, whereas an increased number of lipid bodies with a homogeneous distribution through the cytoplasm were observed during S phase. These data were confirmed with thymidine synchronization. Moreover, NIH3T3 cells showed increased number and dispersed localization of lipid bodies upon transformation with H-rasV12 oncoprotein.

Conclusions: Taken together, these results suggest that lipid bodies are highly regulated during cell cycle, and also that this regulation of lipids is altered in transformed cells. Also, these data provide evidence for a coordinate mechanism that regulates cell cycle progression and lipid body biogenesis, which might be deregulated during cancer development.

[325] Mesenchymal Stem Cells Promote Proliferation of Breast Cancer Cells Via Hyaluronan-CD44 Interaction

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Background: The tumor microenvironment is crucial for cancer cell survival and spreading. Hyaluronan (HA) is a major matrix molecule in the stroma of breast cancer. It is accumulated in about 50% of malignant breast cancer tumors, as well as in bone and soft tissues. However, the origin and exact functions of HA in breast cancer are still unclear. The aim of the study was to explore the role of HA in breast cancer metastasis to the bone marrow.

Material and Methods: Bone marrow derived human mesenchymal stem cells (MSCs) and MDA-MB-468 (parental/Luc) human breast cancer cells were studied in mono- and co-cultures. HA production was analyzed by HA assay and by visualizing HA-rich pericellular matrix with fluorescent HA binding probe. Exogenous high molecular weight HA (LifeCell) was added to the cultures to test HA binding to the cell surface. Binding of HA to its receptor CD44 was blocked with a CD44 antibody (Hermes-1, Developmental Studies Hybridoma bank) and HA was removed from cultures by treatment with pretreated human recombinant PH2 hyaluronidase (PEGPH20). Cell numbers of mono- and co-cultures were analyzed by cell viability and luciferin assay, respectively.

Results and Discussion: MSCs synthesize high amounts of HA, while in MDA-MB-468 cells there are only ~5% HA positive cells. Instead, MDA-MB-468 cells express high levels of CD44 that can bind HA. While co-cultured with MSCs, MDA-MB-468 cells formed distinct pericellular HA coat. Similar HA coats were also observed after addition of exogenous HA to MDA-MB-468 cells. In co-cultures, the HA coat around MDA-MB-468 cells can be prevented by antibody blockade of CD44, showing that formation of HA coat is CD44-mediated. Co-culturing with MSCs increased proliferation of MDA-MB-468 cells similarly. Non-MB-468 cells did not show increased HA-CD44 interaction. In cancer cells, blockade of CD44 has been previously shown to inhibit phosphoinositide 3-kinase (PI3K)-Akt pathway and result in reduced cell survival and drug resistance.

Conclusions: HA produced by MSCs has a major role in MSC-breast cancer cell interaction and may serve as a chemo-attractant for breast cancer cells and promote their proliferation, and potentially metastasis. Since both phenomena seem to be mediated by CD44, interference of HA-CD44 interaction may serve as anti-metastatic treatment in breast cancer. Studies to confirm the importance of HA-mediated MSC-breast cancer cell interaction in tumor growth and spreading in vivo are ongoing.

[326] Repression of Essential Telomerase Components Mediates Anti-proliferative Effects in Isogenic Immortal and Tumorigenic Cells, but not Normal Cells

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Background: The telomere maintenance enzyme telomerase is a ribonucleoprotein enzyme complex, composed of telomerase reverse transcriptase (TERT), the RNA binding and modifying protein dyskerin, and a telomerase RNA component (TERC). Evidence that the individual components of telomerase have functions that extend beyond telomere maintenance provides compelling rationale for direct therapeutic targeting of telomerase components, as ablation of these functions is predicted to have very acute effects that will not be dependent upon telomere shortening.

Materials and Methods: This study compares the functional and molecular consequences of siRNA-mediated repression of TERT, TERC and dyskerin ex-
pression in isogenic normal (MRC5), immortal (pre-malignant) (MRC5×hTERT) and tumorigenic (MRC5×hTERT T2) human myofibroblasts, which are p53-defective, anchorage-dependent fibroblasts cells (HT1080).

Results and Discussion: SIRNA-mediated repression of DKC1 or TERT expression induced acute proliferative arrest of immortal and tumorigenic cells, while having no adverse effect on the proliferation of isogenic normal cells. Furthermore, suppression of DKC1 or TERT gene expression also impaired anchorage-independent growth of tumorigenic cells. In comparison, treatment with a control siRNA, siRNA targeting TERC or the small molecule telomerase enzyme inhibitor riluthiamine (R1532), had no acute effect on proliferation in these short term assays. While the growth arrest mediated by DKC1 or TERT repression was not dependent on p53 function, loss of p53 function altered the cell cycle phase in which the cells arrested. The growth arrest induced by DKC1 repression remained unaffected by TERC overexpression suggesting a telomere independent role for DKC1. Microarray analysis of gene expression and Gene Set Enrichment analysis (GSEA) of siRNA-transfected normal, immortal and tumorigenic MRC5 cells was employed to gain insight to the mechanisms that underpin the acute anti-proliferative effects of TERT and DKC1 repression. Investigations are currently underway to determine the effect of stable repression of TERT and DKC1 on subcuteaneous tumour formation in immunocompromised mice.

Conclusions: Together, these results demonstrate that telomerase-immortalised pre-malignant and tumorigenic cells required continued expression of TERT and DKC1 for replication. This provides further evidence of telomere length-independent functions of TERT and DKC1 in these cells since there was insufficient time for telomere shortening to occur. The potent and specific anti-proliferative effects resulting from repression of DKC1 and TERT in immortal and tumorigenic cells demonstrate that directly targeting these telomerase components has potential as a potent and specific approach to treatment of the broad spectrum of cancers that express telomerase.

329 Role of the Fragile X Mental Retardation Protein in Tumor Progression and Metastasis Formation

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Background: The Fragile X Mental Retardation Protein (FMRP) is responsible for the Fragile X Syndrome (FXS), the most frequent form of X-linked mental retardation resulting from mutations of the Fmr1 gene and affecting 1:2000 males and 1:4000 females.

The protein is widely studied in neurons, where it regulates RNA metabolism acting as a modulator of mRNA stability or translation of mRNA encoding for proteins involved in cytoskeleton remodelling and function of the extracellular matrix.

A correlation between patients with FXS and risk of cancer has been reported. Here, using a mouse model, we investigated the function of FMRP in cancer progression and metastasis formation.

Material and Methods: Using a Tissue Microarray (TMA) we analyzed FMRP expression in a cohort of 477 patients with breast cancer. Functional in vivo and in vitro studies were performed using different murine mammary adenocarcinoma cell lines silenced or overexpressing FMRP. Orthotopic and intravenous injection of those cells allowed us to study the kinetic of tumor progression in vivo. Biochemical and cell biological approaches were used to determine adhesion, invasion and migration of cancer cells with different FMRP levels.

Results: Our findings revealed that patients with breast cancer show an upregulation of FMRP compared to normal tissues. Importantly, in mice, alteration of FMRP level in tumor cells affects the numbers of lung metastasis formation. Furthermore, we were able to highlight that FMRP is not involved in the first step of tumor progression, i.e. intravasation. Finally, we have identified the first FMRP target mRNAs in tumor cells that encode for protein involved in Epithelial-Mesenchymal Transition.

Conclusion: For the first time, an involvement of FMRP in breast cancer progression and in particular in metastasis formation. The cellular and molecular studies, in conjunction with the outcome on the human samples, revealed how a neuronal RNA binding protein affects metastatic dissemination through regulation of mRNAs involved in the early steps of tumor progression.

330 Carbohydrate Response Element Binding Protein (ChREBP) – A New Metabolic Biomarker in Breast Cancer?

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Introduction: ChREBP is a glucose-activated transcription factor which regulates lipogenic enzymes. The purpose of this study was to determine a role for ChREBP in breast cancer progression, and to explore any association between ChREBP, the increased expression of the hypoxia-inducible factor (HIF-1)-inducible facilitative glucose transporter Glut-1 and important pathways within the hypoxic tumour microenvironment.

Method: To examine the link between ChREBP, Glut-1 expression and malignant progression, IHC was carried out in serial sections from two breast cancer tissue microarrays. The first (Pantomics BRC711), included 71 cases of mostly invasive ductal carcinomas; while the second (Cybrid # CC08–00–001) was a breast cancer progression array carrying samples ranging from normal breast tissue through cases of non-malignant hyperplasias and invasive or metastatic carcinoma. ChREBP and Glut-1 staining were scored semi-quantitatively (0 = no; 1 = light, 2 = moderate, 3 = heavy staining). To determine how ChREBP expression may be influenced by hypoxia or activation of the HIF-1 pathway, ChREBP IHC was carried out on a further series of 74 cases of breast cancer samples, for data mining and Spearman rank correlation analysis of an associated cDNA microarray dataset with ChREBP protein and mRNA expression and a 51-gene hypoxia signature or the ‘hypoxia metagenes’.

Results and Discussion: Glut-1 is overexpressed in a wide range of solid tumours, indicates poor prognosis and has been used as a marker of hypoxia. A significant correlation between Glut-1 and ChREBP expression was observed in the Pantomics series, suggesting that Glut-1 overexpression may lead to downstream ChREBP synthesis in invasive ductal carcinoma of the breast (Spearman’s rank correlation r = 0.328 P = 0.013). Analysis of the breast cancer progression array also revealed a clear increase in ChREBP expression with malignant progression; normal breast tissue showing an absence of staining and mild hypoxia showing light staining; whereas locally invasive and metastatic tissue showed the heaviest staining intensity. There was a significant correlation between downregulated hypoxia signature scores (mean gene expression per sample) and both ChREBP protein score (r=0.297, P = 0.0148, n=67) and mRNA expression (r=0.310, P = 1.428e−06, n=74), suggesting a mechanism whereby ChREBP expression is downregulated in hypoxia, or that ChREBP activity interferes with the expression of HIF-1-regulated genes.

Conclusion: ChREBP may be part of an alternative mechanism by which events downstream of amplified Glut-1 expression lead to malignant transformation and progression. In addition, ChREBP may offer a fine-tuning mechanism or means of adjusting the metabolic state in response to fluctuations in oxygenation and HIF-1 signalling.

331 Protein-Coupled Receptor Kinase 2 (GRK2) Contributes to Cellular Transformation and Breast Tumor Progression

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G protein-coupled receptor kinase 2 (GRK2) is emerging as a key, integrative node in many signalling pathways. Besides its canonical role in the modulation of the signaling mediated by G protein-coupled receptors (GPCR), this protein can display a very complex network of functional interactions with a variety of signal transduction partners, contributing to the proper functioning of basic cellular processes such as cell migration or cell cycle progression. Consequently, both expression levels and activity of GRK2 are tightly controlled in normal settings but frequently unbalanced in several relevant pathological contexts like inflammatory, metabolic or cardiovascular diseases and in some tumors, thus suggesting that such changes may be involved in the onset or development of those pathologies. In this context, we find that GRK2 is up-regulated in human transformed epithelial mammary cell lines as well as in the mammary gland of GRK2 over-expressing mice that are prone to develop breast tumors. Interestingly, such up-regulation of GRK2 relies on the over-activation of signaling pathways triggered by ErbB2 receptors or by estrogen receptors in those hormone-responsive breast tumor cell lines. Moreover, accumulation of GRK2 protein seems to contribute to cellular transformation both by mielogenic and antiapoptotic activities of relevant stimuli in breast tumor progression such as epidermal growth factor (EGF) and heregulin. On the contrary, GRK2 knock-down in breast cancer cell lines results in suppression...
The Microenvironment Regulates Responses to Hepsin Overexpression in Prostate Cancer Cells

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Introduction: The serine protease hepsin was found to be overexpressed in approximately 90% of prostate cancer cases. Conversely, ectopic overexpression of the enzyme was described to suppress viability and invasive growth in cell lines of prostate, ovarian, and endometrial cancer origin. Although hepsin was demonstrated to contribute to matrix degradation and invasive growth of prostate cancer in vivo, this apparent paradox raised questions about the role of the enzyme in cancer cells.

Material and Methods: We employed doxycycline (dox)-inducible overexpression of hepsin in PC3 prostate cancer cells to analyze cellular effects of the enzyme in a quantitative manner. Subsequent to graded induction of the gene in different microenvironments, cells were analyzed for viability, adhesion and the expression / phosphorylation state of signalling proteins using flow cytometry, cell-based assays, and Westernblot analysis.

Results and Discussion: We observed a gradual loss of cell adhesion and viability with increased expression levels of hepsin during growth in conventional cell culture dishes and on extracellular matrix (ECM) secreted by PC3 cells. Adhesion and viability was partially restored during growth on ECM secreted by non-tumorigenic RWPE1 cells. During anchorage-independent growth, cell viability did not correlate with expression levels of hepsin. Dephosphorylation of AKT at serine473 correlated tightly to hepsin overexpression in these cells, but could be pharmacologically reversed by growth on RWPE1-derived ECM. Thus, ECM secreted by non-transformed prostate epithelial cells seems to provide a protective microenvironment for hepsin-overexpressing prostate cancer cells.

Conclusions: Our findings suggest that expression levels of hepsin must be spatially and temporally restricted for the efficient development of tumours and metastases. Modulation of the microenvironment may therefore be a promising therapeutic option for hepsin-positive prostate tumours.

A New CXCR4 Receptor Antagonist – Effects on Cell Growth and Tumor Microenvironment in a Glialoma Model

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Background: The chemokine receptor CXCR4 is a widely expressed G protein-coupled receptor. Although initially linked with leukocyte trafficking, CXCR4 is expressed in various tumors, where it is strictly related to cell motility, proliferation and survival. In gliomas, CXCR4 activation by the agonist CXCL12 has been demonstrated to regulate the recruitment of the surrounding microglia/macrophages. Therefore, the CXCL12/CXCR4 signaling pathway is emerging as a potential therapeutic target. In this study we evaluated the effects mediated by a new CXCR4 receptor antagonist, the cyclic peptide Phe-7, compared with AMD3100, a well-known CXCR4 inhibitor, on a human glioma cell line (U87MG), and the influence of these drugs on microglial reactivity, by using intracranial xenografts.

Material and Methods: U87MG cell line was obtained from ATCC and for in vivo experiments cells were stereotaxically implanted into the caudate nucleus of CD1 nude mice then separated in: control group (PBS-treated), AMD3100- and Phe-7-treated groups. Treatments were administered twice daily (for 15–20 days) by i.p. injection since U87MG cells implantation. To monitor tumor growth, tumor volume was assessed by MRI analyses, performed at 4.7 T on VARIAN Agilent system (Agilent, Palo Alto, US). Mice were then perfused and brains were fixed and frozen following standard protocols. Sections were stained for different tumor and microglial markers and analyzed by confocal laser scanning microscopy (CLSM). The effects of Phe-7 and AMD3100 on U87MG cells were investigated by using CLSM, flow cytometry, MTT assays and Western blot analyses.

Results and Discussion: The expression of CXCR4 was strongly down-regulated after treatment of U87MG cells with Phe-7 within 48–72h. Cell viability (MTT) and cell count assays showed a Phe-7-mediated decrease (about 40%) in the amount of living/proliferating cells after 72h of incubation, compared to control cells. Moreover, in these experimental conditions neither apoptosis nor necrosis were induced, thus suggesting a possible block of cell proliferation. Immunohistochemistry performed on brain sections of the Phe-7-treated group, showed a strong reduction of CD11b+ cells (macrophages/microglia markers) at the tumor edge, together with an increase of CD11b+/iNOS+ cells (inducible nitric oxide synthase as a marker for the pro-inflammatory phenotype of microglia) in the tumor core. While, in the untreated gliomas a strong expression of arginase-1 at the level of endothelial cells was observed, together with a pool of CD11b+/iNOS+ cells in perivascular areas. These features were not observed in the Phe-7-treated tumors.

Conclusions: These data indicate that the new CXCR4 receptor antagonist, the peptide Phe-7, could modulate glioma-microglia interactions mediated by the CXCR4/CXCL12 signaling axis, thus interfering with microglial recruitment and activation.

Myc/p27 Balance in Chronic Lymphocytic Leukemia

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Background: Chronic lymphocytic leukemia (PLL) is the most common leukemia in the western world. CLL is characterized by the accumulation of CD5+ B lymphocytes. However, clinical outcome of CLL may be different. Some patients have an indolent leukemia with long survival while others experience an aggressive disease. Contrary to other human malignancies, the cyclin-dependent kinase inhibitor p27kip1 (p27) has been described to be overexpressed in CLL cells. On the other hand, Myc is an oncogenic transcription factor overexpressed in many human tumours. In recent years, work in several cellular models have demonstrated that Myc, through different mechanisms, antagonize p27 expression and its anti-proliferative activity. Thus we set out to study the interaction functional between p27 and Myc in B-CLL and whether changes in p27/Myc ratio correlated with the clinical features of the disease.

Material and Methods: Protein and mRNA levels of Myc and p27 in CLL patients and Mec1 cells were analyzed by Western Blot and real time RT-PCR. We studied p27 and Myc localization by immunofluorescence. Annexin assay was performed to study the fludarabine resistance. SPSS and GraphPad Prism were used for statistical studies.

Results: We studied expression levels of p27 and Myc in more than 100 CLL patients using peripheral blood, tonsil and CD19+ lymphocytes as control. p27 and Myc levels were inversely correlated. Thus, the ratio between p27 (overexpressed) and Myc (downregulated) appears inverted in CLL with respect to the other tumours so far known. Moreover, low p27 and high Myc expression correlated with the expression of Skp2, a subunit of ubiquitin ligase complex SCF which is the main responsible for p27 degradation. Skp2 is a Myc target gene and promotes the degradation of p27, suggesting a pathway Myc→Skp2→p27 in CLL. To investigate why p27 is expressed in CLL we overexpressed in a CLL-derived cell line (Mec1) and observed that high levels of p27 provide resistance to fludarabine treatment. In fact, p27 expression was strongly correlated with Bcl2 levels, an antiapoptotic protein, in CLL samples.

Conclusions: Our data provide new insights into the p27/Myc balance in CLL cells. The high percentage of p27 overexpressed patients suggests an important function in CLL, causing low levels of Myc and increasing resistance to apoptosis, likely through collaboration with Bcl2. These results suggest a functional explanation so as why p27 is overexpressed in CLL.

Changes in Bcl-2 Protein Expression Regulate Sensitivity to Chemotherapy Drugs in Multi-cellular Tumour Spheroids

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Most chemotherapy drugs work through the induction of apoptosis in tumour cells. Mutations in apoptotic pathways, notably p53 and Bcl-2, are common in cancer cells and associated with drug resistance. In order to study the resistance to apoptosis and therefore to chemotherapy. We have compared the sensitivity of two cell types, A172 human glioblastoma cells and MDA-MB-231 human breast cancer cells, for their sensitivity to the chemotherapy drugs cisplatin, doxorubicin, gemcitabine, vincristine and temozolomide. We have then determined the effect of growing these cells as two-dimensional monolayers or as three-dimensional multi-cellular spheroids on their sensitivity to these drugs and correlated changes in intrinsic sensitivity with changes in the morphology of the Bcl-2 family of proteins. We found in both cell lines that growing the cells as multi-cellular spheroids significantly enhanced their resistance to apoptosis induced by each of the chemotherapy drugs when compared to
Inhibition of P90RSK Sensitizes Ovarian Cancer Cells to Apoptosis

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Ovarian cancer is the leading cause of death among gynecologic cancers. In ovarian cancer, apoptosis is impaired by the anti-apoptotic action of several oncoproteins, including growth factor receptors and other kinases. The MET oncogene is overexpressed in a consistent fraction of ovarian cancers. Its ligand HGF has been found in ovarian cancer ascitic fluids and in fluid of benign and malignant ovarian cysts. However, it was previously shown that HGF might sensitize ovarian carcinoma cells to the chemotherapeutics agents. We have carried out the search for signal transducers of the pro-apoptotic signalling triggered by the MET receptor. We found that long-term MET activation switched off the activation of the p90RSK triggered by apoptotic stimuli. A crucial role of this protein family has been reported in oncogenesis and tumour progression, so that p90RSK is considered an attractive therapeutic target for cancer treatment.

Material and Methods: Western blot analysis was carried out to evaluate the phosphorylation of ERK1/2 and p90RSK in cells committed to death by the treatment with or without the ERK1/2 inhibitor 49126 for 48 hours in without cisplatin. Cell survival after exposure to the RSK specific and potent inhibitor BI-D1870 was evaluated by staining cells with Annexin V/DAPI in a panel of ovarian cancer cell lines (SKOV-3, IGROV-1, OV-90, TOV21G, OAW42, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8 and OVCAR-4) in HUVEC primary cell line, immortalized human cell lines (MCF10A, 293T), and other tumour cell lines (GTL16, HELA).

Results: Treatment of ovarian cancer cells with HGF for 48 hours abrogated the phosphorylation of p90RSK at the Ser386 elicited by cisplatin. The simultaneous loss of the phosphorylation of its substrate BAD showed that the kinase activity and thus likely the anti-apoptotic activity was impaired. The switch off of the p90RSK in ovarian cancer cells committed to death also suggested that in these cells p90RSK might be indispensable for survival. BI-D1870 did not affect the viability of primary and immortalized non-transformed cell lines. On the contrary, the panel of ovarian cancer cell lines could be subdivided in three different groups according to their BI-D1870 susceptibility: resistant, intermediate and sensitive cell lines. Moreover, a further decreased viability was observed when sensitive and intermediate resistant cells were pretreated with BI-D1870 for 2 hours and then treated with cisplatin for 48 hours.

Conclusion: In conclusion, p90RSK could be regarded as a suitable target for therapy in either mono-therapy or in combination with chemotherapeutic drugs.

Expression of Snail 1 in the Progression of Prostate Cancer

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Background: During the progression of the tumor cells lose their acinar organization and intercellular contact between acinar cells (CoA) is weak, and the lumen is wider than normal. However, the CoA is maintained until the formation of a transitional cell in the luminal border of the acinar epithelium. Snail 1 (Snail1) is a zinc-finger transcription factor involved in the repression of E-cadherin and induction of mesenchymal transition. Our aim is to study the expression of Snail 1 mRNA in prostate cancer samples and correlate it with epidemiologic data regarding the risk factors of this disease.

Materials and Methods: We obtained biopsies of patients with PCa and benign prostatic hyperplasia (BPH) for routine diagnosis of prostate cancer. We analyzed samples by reverse transcriptase-polymerase chain reaction (RT-PCR) using the human Snail 1 primer set. A human Snail1 cDNA fragment was obtained from the pMTG vector, a Snail1 expression vector containing a modified human Snail1 cDNA. A first-strand cDNA was synthesized from 0.5 mg total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies). The PCR products were sequenced with the BigDye Terminator version 3.1 Cycle sequencing kit and the ABI 3100 genetic analyzer. The mRNA expression of Snail 1 was calculated as the mean of the experiment.

Results: Snail 1 expression was significantly higher in PCa samples (p < 0.05) and the average of Snail 1 mRNA expression in the BPH group was 1.36 (0.31) and in the PCa group was 2.98 (0.46). A significant difference was found between BPH and PCa samples (p < 0.05).

Conclusion: A significant higher expression of Snail 1 was found in patients with prostate cancer. This expression could be useful for identifying patients with a higher risk of adverse outcomes of prostate cancer.

The HER2 Amplification Includes Several Genes Required for the Growth and Survival of HER2 Positive Breast Cancer Cells

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Background: About 20% of breast cancers are characterized by amplification and overexpression of the HER2 oncogene. Although significant progress has been achieved for treating such patients with HER2 inhibitor Trastuzumab, more than half of the patients respond poorly or become resistant to the treatment. Since the HER2 amplification at 17q12 contains multiple genes, we have systematically explored the role of the HER2 co-amplified genes in breast cancer cell growth and their relation to Trastuzumab resistance.

Materials and Methods: We integrated aCGH data of the HER2 amplicon from 71 HER2 positive tumors and 10 cell lines with systematic functional RNA interference analysis of 23 core amplicon genes of Trastuzumab responding and non-responding HER2 positive breast cancer cell lines. Several key signaling components were used as screening readouts by utilizing protein lysate microarray technology.

Results: Sensilicating of HER2 caused a greater growth arrest and apoptosis in the responding compared to the non-responding cell lines, indicating that the resistant cells are inherently less dependent on the HER2 pathway. Several other genes in the amplicon also showed clear effects on survival when silenced; indicating that expression of HER2 co-amplified genes may be needed to sustain the growth of breast cancer cells. Importantly, co-silencing

Reference(s)

of ampliton genes together with HER2 or co-treatment with Trastuzumab / Lapatinib led in many cases to synergistic inhibition of cell viability.

Conclusions: These studies indicate that breast cancer cells may become addicted to the amplification of some genes that reside in the HER2 amplicon. Simultaneous targeting of these genes may increase the efficacy of anti-HER2 therapies and possibly also counteract Trastuzumab resistance.

[335] Wnt/Beta Catenin Pathway Differences After Treatment of Primary and Metastatic Colon Carcinoma Cell Lines

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Background: The members of Wnt family play a role during cell polarity, proliferation, apoptosis and cell migration during both embryonic and pathologic conditions. Signaling of Wnt is control with two different pathways; the first one that the activation of Dishevelled (Dvl) via occurring of Wnt/Fzd complex after triggering of β-catenin into nucleus for starting of transcription. The second one that, it is β-catenin independent pathway and activate profillin, Rac and Rho and then cytoplasmic pathways for cell differentiation is controlled. Because of the role of the Wnt signaling pathways during epithelial-mesenchymal cell interaction, it is important for during occurring or metastasis of cancer cells. In our study, we aimed that the Wnt/β-catenin pathway differences after treatment of primary and metastatic colon carcinoma cell lines.

Material and Methods: Primary (Colo-320) and metastatic (Colo-741) colon adenocarcinoma cell lines were used. They were cultured in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 1% L-glutamin and 1% penicillin-streptomycin. After 1 week of culture, the gastrointestinal cell lines were divided four groups. Groups 1 were not treated any medicine. Groups 2, 3 and 4 were treated with sulindac, α-lactaalbumine and sulindac+α-lactaalbumine, respectively. After 48 hours of treatment, the cells were fixed with 4% paraformaldehyde and the distribution of Wnt-7a, β-catenin, Dkk-1, Dkk-3 and Fzd-6 were analyzed using immunoperoxidase staining.

Results: The Colo-320 cells were semi-adhesive cells; the Colo-741 cells were attachment cells. The immunoreactivity of Wnt-7a was less in all groups, immunoreactivity of β-catenin was more in Colo-741 cell lines than Colo-320 cell lines in all groups. The immunoreactivity of Dkk-1 was similar in both cell types, however, the intensity of Dkk-1 was strongly observed after treatment with sulindac. The Frizzled-6 immunoactivity was also detected in both cell lines. Conclusions: Our results supported that the Wnt/β-catenin pathway was differently activated in both primary and metastatic colon carcinoma cells after treatment. Especially sulindac was more triggered pathway in Colo-320 cells rather than Colo-741 cells. During cancer therapy, the correct time and correct medicine is crucial for different patients. Because of cancer cell behavior, treatment of the primary and metastatic colon cancer may also be different for each patient.

Detection of IL-1β, IL-6 and TNF-α in Cultures of Primary Gastrointestinal or Breast Cancer Cells After Treatment

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Background: Both gastrointestinal and breast cancers are the most frequent malignancies and the development of cancer sometimes associated with chronic inflammation. The most convincing examples of chronic inflammation-induced carcinogenesis are seen within the gastrointestinal tract. In addition, the link between inflammation and carcinogenesis during development and after treatment are not well understood. Prognoses after treatment may depend on the person and also expression of different interleukins and/or cytokines.

Material and Methods: Gastrointestinal and breast tissues were collected during surgery and they were transferred in sterile culture medium into the laboratory. They were sliced with steril blade and treated with trypsin/EDTA solution for 4 hours at 37ºC, 5%CO2 in air. After the fresh culture medium were added to cells for each plates, the medium collected and centrifugation. The cells were then cultured in RPMI-1640 medium with 10% fetal bovine serum, 1% L-glutamin and 1% penicillin-streptomycin. After 1 week of culture, the gastrointestinal cancer cells were treated with 5-FU and irinotekan, breast tumor cells were treated with 5-FU, epirubisin and cisplatin. Treatments for both tumor types were applied for 24, 48 and 72 hours. The cells were collected and the level of M30, M65, TNF-α, IL-1β and IL-6 were analyzed using ELISA.

Results: In both cell types, cytotoxicity was observed and cell death was also detected after treatment. Analysing of both M30 and M65, the values of M65 were higher in some cases, therefore, in that cases, the necrotic pathway may be triggered after treatment. The values of TNF-α were same in all cases, however, the values of IL-1β were different in some cases after treatment with different medicine. The values of IL-6 were observed very high in all cases.

Conclusions: In both gastrointestinal and breast tumor cells which obtained from primary tumor tissues in 6-8 days period can be suitable to decide specific protocols for therapy for different patients considering different parameters. Standard therapy protocol may be revising according to differences of cells.

[341] Carcinoma Associated Fibroblasts Provide a Niche for Cancer Stem Cells - Analysis of a New Cell Culture Model in Urothelial Cancer

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Background: Tumours are organized in a hierarchical manner, with cancer stem cells (CSC) at the top of this intrinsic cellular hierarchy. Mounting evidence indicates that stromal cells contribute crucially to the regulation and maintenance of CSC during tumour development and progression. However, experimental systems to study this niche-providing activity of cancer stromal cells are relatively scarce.

Material and Methods: Cell lines were established according to our published protocol (Skeie et al., World J Urol. 2007 Jun;25(3):297–302). CAFs were immortalized by retroviral transduction of the hTERT gene. Differentiation activity of immortalized CAFs towards osteogenic, chondrogenic and adipogenic lineages was assayed by standard protocols. Expression of selected CSC antigens was analyzed by indirect immunofluorescence staining.

Results: We report establishment and characterization of a pair of carcinoma (BC44) and carcinoma-associated fibroblast (BC44Fibr) - cell lines derived from the same urothelial tumour. BC44Fibr cells display typical attributes of carcinoma-associated fibroblasts (ubiquitous expression of Vimentin and Smooth Muscle α-Actin, prevalent expression of Fibroblast Activation Protein). Focal expression of CD13 suggests their bladder stroma origin, nevertheless, the cells could be successfully differentiated into osteocytes and chondrocytes, a property of cells originating from tumour-recruited mesenchymal stem cells. Co-culture of BC44Fibr and bladder carcinoma cell lines (BC44, SW780, HT1197) revealed that carcinoma cells strongly positive for putative CSC markers (Cytokeratin 17, 67 KDa Laminin Receptor, CD44v6) were located at the very margin of carcinoma cell colonies, i.e. in direct contact with co-cultured carcinoma fibroblasts. The stem cell promoting activity of BC44Fibr cells manifested even in co-culture with normal skin keratinocytes.

Conclusion: We believe that this experimental system could be very valuable in deciphering the mechanisms involved in stem cell promoting activity of carcinoma fibroblasts.

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[342] Hydroxyicosatetraenoic Acids Are Involved in Intestinal Epithelial Cancer Cell Growth

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Background: Cyclooxygenase (COX) and 5- lipoxigenase (5-LOX) are simultaneously up-regulated in human colorectal cancer [1] and a remarkable expression of leukotriene B4 receptor (BLT1) and cystein LT receptor (CysLT1) were also detected in human colon cancer tissues [2,3]. However few information is suitable about the role of hydroxyicosatetraenoic acids (HETEs). For this reason, we aimed to study the role of these eicosanoids derived LOXs-pathway in the control of Caco-2 cell growth.

Material and Methods: We measured the biosynthesis of HETE by intestinal epithelial cell cancer (Caco-2 cells) using liquid chromatography/tandem mass spectrometry and studied the effects of HETEs on Caco-2 cell growth and DNA synthesis.

Results: We observed that intestinal epithelial Caco-2 cells cultured in the presence of 10% fetal bovine serum produce 5-HETE (8.7±2.06 nM), 12-HETE (4.89±2.13 nM) and 15-HETE (7.96±1.53 nM). Exogenous addition of 5-HETE, 12(5)-HETE and 15-HETE (10–100 mM) were able to induce Caco-2 cell growth and [3H] thymidine incorporation in absence of another growth factor. HETEs receptors have not been identified but our results showed that U75302 (BLT1 antagonist) and LY255283 (BLT2 antagonist) were able to inhibit Caco-2 cell growth induced by HETEs. Moreover, the proliferative effect of HETEs was blocked by ketooprofen, a COX inhibitor as well as SC19088. Therefore, we observed that effects of HETEs on Caco-2 cell growth was mediated by COX and inhibitors.

Conclusions: These findings suggest the role of HETEs on intestinal epithelial cell growth through the interaction with BLT receptors and the induction of PGE2 synthesis.

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Reference(s)

Analysis of Cancer Associated Fibroblasts in Tumor Microenvironment

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The tumor environment consists of endothelial cells, activated fibroblasts, inflammatory cells as stromal cell components, and extracellular matrix. Angiogenesis, attracted attention in the cancer therapy, but these effects are insufficient to completely kill all of the cancer cells. Blood vessels are composed with endothelial cells (ECs) and mural cells (MCs) in normal tissues; however, ECs are not fully covered with MCs in the tumor environment and MC like cells alone can generate capillary like structure called ‘ghost vessels’. PDGFRβ is known as vessel-associated pericyte and fibroblast markers.

We recently found that stromal cells expressing PDGFRβ were abundantly observed in tumor environment after treatment of low dose anti-cancer drugs and those stromal cells generated capillary like structure. Therefore it is possible that such stromal cells function as pipes like blood vessels and supply oxygen and nutrient to tumor tissue after treatment of angiogenesis disrupting agents. Here, we show the isolation and characterization of tumor stromal cells, cancer associated fibroblasts (CAFs), obtained from B16 melanoma tissue. Most of CAF cell lines express PDGFRβ, β1, α5, α3, CD34. We identify the condition of how such CAFs generate tube like structure in vitro. Now, we are attempting to identify novel target molecules for tube formation of stromal cells. Therefore we generated monoclonal antibody (Mab) by immunization of CAFs. We obtained a Mab against a certain factor, which tumor stromal cells highly expressed under hypoxic mima on condition. They are attempting to identify novel roles of the molecules in the tumor environment. The Mab specifically recognizes mouse molecule, as evidenced by immunoblotting using the cell lysates. Additionally, in flow cytometric analysis, the Mab detects, mouse one but not human one. Furthermore, in immunohistochemical staining, the Mab stains only a part of the mouse. These results suggested that this antibody would provide an effective tool to analyze host (mouse) cells in human cancer cell xenograft model in vivo.

The MUC1 Membrane-bound Mucin is an Actor in Renal Clear-cell Carcinoma

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Introduction: MUC1, an O-glycoprotein membrane-bound mucin, is overexpressed in renal clear-cell carcinomas (CRCC) with correlation to two major prognostic factors, Tumor-Node-Metastasis stage and nuclear Führman grade. Previously, we have shown that (i) MUC1 was significantly overexpressed in metastatic CRCC vs non-metastatic CRCC and (ii) MUC1 is a target gene of HIF-1 transcription factor which is a part of the hypoxia pathway, the main renal carcinogenetic pathway.

Material and Method: To better understand the roles of MUC1 in CRCC, we studied two cell lines expressing MUC1 (786-O cells) or not (ACHN cells). 786-O cells were stably transfected with shRNA targeting MUC1 while ACHN cells with full-length MUC1, tandem-repeat deleted or cytoplasmic tail truncated construct cDNA. Proliferation, drug resistance, migration and invasion properties were studied in vitro in the different cellular clones using MTS cell proliferation assay, wound healing assay and Boyden chambers coated with Matrigel, respectively. Signaling pathways were screened by western blot analyses. Xenografts were performed in SCID mice.

Results and Discussion: We showed that MUC1 expression was associated with increased invasion and migration properties of renal carcinomatous cells and a decrease of cell-cell interactions whereas no effect on proliferation was observed. These effects were dependent on MUC1 tandem-repeat domain and cytoplasmic tail. MUC1 overexpressing cells (i) expressed higher levels of NF-κB transcription factor and two genes involved in chemoresistance processes and (ii) were more resistant to chemotherapeutic drugs. MUC1 expression accelerated xenograft tumor growth in vivo.

Conclusion: Our results show that MUC1 plays a role in biological properties of renal cancer cells suggesting important function for this mucin in tumour progression and confirm its potential as a therapeutic target in this type of cancer.

Sedanolide Induces Human Liver Tumor Cell Autophagy Through Regulation of NF-κB Pathway

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Background: Sedanolide, an active component of Oenanthe javanica (Blume) DC, has shown antioxidant potential in vitro and ex vivo. However, the regulation mechanisms [DB1] that sedanolide induces autophagy is limited.

Material and Methods: In this study, cultured human liver tumor cells (J5) was used as the experimental model to investigate the effects of sedanolide autophagy and signal pathways.

Results: According to the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, the cell viability of J5 cells was significantly decreased, whereas J5 cells was treated with 500 μM sedanolide for 24 hours (P < 0.05). The formation of autophagic vacuoles in the cells treated with 100, 250, or 500 μM sedanolide after 24 hours were observed by fluorescent microscopy of monodansylcadaverine staining. Beclin-1 and LC3-II protein expressions were significantly increased after 500 μM sedanolide treatment for 24 hours (P < 0.05). In addition, the NF-κB signaling pathway were activated through an increase of phosphorylation of IκB and DNA binding activity of NF-κB after 500 μM sedanolide treatment in J5cells (P < 0.05).

Conclusions: These results have demonstrated that sedanolide induces human liver tumor cell autophagy via activating NF-κB signaling pathways.

Role of GRK2 in Developing Vasculature and Pathological Angiogenesis

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G protein-coupled receptor kinases (GRKs) were initially identified as key players in the desensitization and internalization of multiple G protein-coupled receptors (GPCR). Besides such traditional role in GPCR signaling, recent data indicate that GRKs can also initiate alternative signaling pathways and participate in a variety of cellular processes. GRK2 can phosphorylate a growing number of non-GPCR substrates and associate with a variety of proteins related to signal transduction, thus displaying novel ‘effector’ functions that have been shown to underlie its participation in key cellular processes such as cell cycle, insulin resistance or cell migration. Consistent with such basic impact on cellular physiology, altered GRK2 expression has been linked to several cardiovascular disorders as well as to inflammatory and neoplastic condition. Interestingly, we have found that embryonic lethal GRK2 knock-out mice display defects in embryonic angiogenesis and vasculogenesis consistent with an inefficient maturation of vessels, which suggest a critical role for GRK2 in vascular remodeling during development. By using primary cultured cells from wild-type and GRK2 hemizygous mice, we confirm that GRK2 modulates signaling, proliferation and migration of endothelial cells to different relevant physiological stimuli. Expression of GRK2 is also critical for in vitro tubule formation and for barrier function of the endothelium. Accordingly, in vivo neo-vascularization and vessel maturation was altered in both global and endothelium-specific GRK2 knockout mice. Finally, we have shown that endothelial expression of GRK2 is downregulated at both protein and mRNA level during tumour angiogenesis, but not in normal angiogenesis. Such downregulation leads to formation of more immature vessels, with a markedly defective investment of pericytes. The extent of neoplastic growth inversely correlates with the dosage of GRK2 in the endothelial vascular component. Overall, our results suggest that endothelial downregulation of GRK2 could play a relevant role in creating a permissive microenvironment for tumour progression.

Evaluation of Gefitinib Maintenance in an EGFR-mutant NSCLC Cell Line With Acquired Resistance

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Background: Lung cancers harboring mutations in the epidermal growth factor receptor (EGFR) respond to EGFR tyrosine kinase inhibitors, but drug resistance invariably emerges. Two major resistance mechanisms have now been revealed. T790M secondary mutation increases the affinity of the oncogenic mutant EGFR for ATP, and this leads to the reduced efficacy of EGFR-TKIs. Almost half of the patients with acquired resistance seem to have this mutation. MET amplification is another mechanism to escape to anti-tumor effect of EGFR-TK inhibitors, which allows cell survival by persistent Akt signaling. However, there are indications suggesting that EGFR-mutant lung cancer maintains sensitivity to TKIs after development of resistance and patients can still be sensitive to re-treatment with TKIs. To date, a standard therapy for the treatment of NSCLC after progression to TKIs is lacking. Considering these data we hypothesize that EGFR-mutated patients could benefit from a second-line therapy based on the peculiar characteristics of sensitivity shown by tumor cells.

Given this background we investigated the effect of continuation of gefitinib in resistant gefitinib cell lines.

Material and Methods: Experiments were performed on HCC827GR5 NSCLC cell line (kindly provided by Dr. P. Janne Dana-Farber Cancer Institute Boston MA) carrying NSCLC mutation but without acquired resistance to gefitinib (RTK). Progenitors were established using Boyden chambers, adhering transfection and migration were evaluated by Western Blotting.

Results and Discussion: Gefitinib withdrawal did not modify resistance phenotype regarding the angiogenesis index and response to the drug in respect to cells continuously maintained in the presence of the drug. On the contrary, in the absence of gefitinib, resistant cells showed more migrated and invasive capability, with activation of the Src pathway. In addition they showed typical EMT markers. The prolonged treatment with gefitinib, instead, reduced cell migration, cell invasion and mesenchimal markers.

Conclusion: These results indicate that cells which have become resistant to gefitinib can proliferate and survive in the presence of the drug, however the maintenance of gefitinib might be important to control other malignant phenotypes of tumour cells such as loss of epithelial features and the acquisition of invasiveness.

536 Angiogenin II Promotes Head and Neck Squamous Cell Carcinoma Progression by Modulating Stromal-epithelial Groustalk
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Background: Angiogenin II (Ang II) is the product of the proteolytic action of angiotensin converting enzyme (ACE) on the precursor peptide, Angiotensin I (Ang I). In addition to its vasoactive properties, Ang II is able to stimulate angiogenesis and act as a mitogen, promoting cellular proliferation; these effects are predominantly mediated through the angiotensin 1 receptor (AT1R). Blockade of AT1R has been demonstrated to inhibit angiogenesis and cancer cell proliferation in a number of tumour types. Recently, evidence has emerged that Ang II can also promote tumour invasion, a key step in the metastatic cascade. The mechanisms by which it does so remain largely obscure. It is becoming increasing apparent that the tumour microenvironment contributes to cancer progression. The signals that control stromal-tumour interactions remain poorly understood. Here we investigate the role that Ang II plays in the expression of phenotypic markers associated with the transdifferentiation of HNSCC cells.

Material and Methods: The effect of Ang II on head and neck squamous cell carcinoma (HNSCC) cell lines were investigated using 2D migration assays and 2D Matrigel assays. Quantitative PCR was used to determine the expression profiles of components of the renin-angiotensin system within a panel of primary human normal oral keratinocytes (NOKs) and fibroblasts (NOFs) and cell lines derived from oral dysplasias, primary HNSCCs and a local metastasis. The effect of Ang II on the migration and proliferation of primary human NOFs was investigated using scratch and MTS assays, respectively. The contractile phenotype of NOFs after treatment with Ang II was assessed using type-1 collagen gels and quantitative PCR was used to assess the expression of phenotypic markers associated with the transdifferentiation of NOFs.

Results: Ang II is able to promote the invasion and migration of HNSCC cells in an autocrine manner and promotes NOFs activation, triggering stromal-tumour interactions. This effect is mediated via AT1R which is highly expressed by both HNSCC cells and NOFs, but not by NOKs. Ang II increases the proliferation and migration of NOFs and induces a more contractile phenotype. The effects of Ang II on autocrine and paracrine signalling pathways are inhibited by angiotensin 1-7 (Ang 1-7), a peptide produced from Ang II by the action of angiotensin converting enzyme 2 (ACE2).

Conclusion: These data are the first to demonstrate a role for the renin-angiotensin system in head and neck carcinogenesis, a frequently fatal and increasingly common epithelial malignancy of the oral cavity. It raises the possibility of utilising AT1R antagonists and/or Ang 1-7, a potential therapeutic agents for HNSCC. The ability of Ang II to modulate the phenotype of human NOFs reiterates the importance of the relationship between HNSCC cells and the surrounding tumour microenvironment.

537 Statistical Analysis of the 21 Cases of Cutaneous Angiosarcoma Treated in the Past 5 Years at the Ryukyu University Hospital
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Background: Cutaneous angiosarcoma (AS) is a malignant tumor of the vascular system, characterized by purpuritic plaques, nodules, or ulcerations on the head or face in the elderly. In Japan, this tumor is extremely rare and has an extremely poor prognosis, a 10−15% 5-year survival rate. The standard treatment strategy for cutaneous AS has not been established.

Subjects and Methods: Subjects and Methods: Using statistical analysis, we determined the survival time and prognostic factors for 21 patients with cutaneous AS who were treated between June 2006 and May 2011 at the Ryukyu University Hospital.

Results: The 5-year survival rate for the 21 patients was 23%. Age (P = 0.02), presence of distant metastasis (P = 0.03), and size of the lesion (P = 0.04) were identified as significant prognostic factors. Tumor size at the time of initial diagnosis was 79.7 years. The patients comprised of 13 men and 8 women. Three patients developed cutaneous AS after injury. Of the 14 mortalities, 12 were due to lung metastases. The mean survival time after initial diagnosis at our hospital was 5.8 months. Radiation therapy was performed for all patients, and chemotherapy with docetaxel was administered to all the patients who were younger than 80 years and had no metastasis. Although survival rates of patients who did and did not receive immunotherapy with interferon-2 (IL-2) were not significantly different (P = 0.64), 2 patients who received surgical treatment, chemotherapy, and immunotherapy survived for 5 or more years.

Conclusions: In this analysis, age, size of the lesion, and presence of distant metastasis were identified as potential prognostic factors for cutaneous AS. Immunotherapy with IL-2 did not significantly influence the survival rate. Cutaneous AS frequently occurs in elderly persons; therefore, in many cases of this tumor, aggressive treatment may not be recommended, and radiation therapy may be the only suitable treatment option.
A Twist1 Code of P53 Inactivation
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Twist proteins have been shown to contribute to cancer development and progression by impinging on different regulatory pathways, but their mechanism of action is poorly defined. By investigating the role of Twist in sarcomas, we identified an unprecedented mechanism of destabilization of p53.

We show that Twist1, by its C-terminals called Twist box, bounds the C-terminal regulatory domain of p53 and physically hinders p53 key phosphorylations facilitating MDM2:p53 complex formation and p53 degradation. This study suggests the existence of a Twist code of p53 inactivation in sarcomas and provides the proof of principle that targeting the Twist:p53 interaction may offer additional avenues for cancer treatment.

Natural Killer Cell Responses to Tumor Priming
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Background: Human Natural Killer (NK) cells are lymphocytes that play an important role in host protection against viral infections and tumors. Our group previously hypothesized a two-stage process for resting NK cell activation. A ‘priming’ signal can be delivered by an activating cytokine such as IL-2 to generate a lytically-activated NK cell (LAK). Similarly, conjugation to a tumour cell expressing an appropriate intensity and combination of ligands, can also result in NK cell priming and the generation of a tumour-activated NK cell (TaNK). The second ‘triggering’ signal is more specific to prevent auto-reactivity, and initiates NK cell degranulation and/or cytokine release. The CTV-1 leukemic cell line is capable of priming NK cells to kill NK-resistant tumours. In this set of experiments we have investigated NK cell responses to tumour priming/cytokine-primed NK cell surface expression, cytotoxic capacity and secretome profiles compared to cytokine-primed NK cells.

Methods: Human peripheral blood mononuclear cells (PBMCs) were isolated from consenting normal healthy donors by density gradient separation and CD56+ purification was performed using magnetic microbeads. NK cells were incubated overnight with whole CTV-1 cells, IL-2, IL-7, IL-15, IL-12 or IL-21. Using 8 colour flow cytometric immunostaining NK cell receptor expression was analyzed. The NK cell secretome was examined using the cytokometric bead array (CBA) assay and the CD107a assay was performed to determine NK cell degranulation.

Results and Discussion: Tumour-priming of NK cells is equivalent to cytokine-priming as demonstrated by the upregulation of the activation markers CD69 and CD25, as well as the enhancement of NK cell cytotoxicity and cytokine production. However, NK cell responses to in vitro stimulation by CTV-1 appeared different to all the cytokines tested. Uniquely, TaNKs upregulated the inhibitory receptor NK2CA and downregulated the activation receptors NK2GD, DNAM-1, ICAM-1, NKp44 and NKp46. Moreover, NK cell subsets defined phenotypically by their CD56, CD2, CD56 and CD16 expression, responded differently to the various stimuli in terms of their activation marker expression, cytokine production and degranulation.

Conclusion: Tumour-mediated priming of NK cells is different from cytokine-mediated priming as evidenced by unique cell surface protein expression patterns, secretome profiles and killing capacities.

Clonogenicity and in Vivo Growth Identify Two Types of Stem-like Giblotastoma Cells (SGLC) Differing in Tumorigenicity and Invasiveness
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Introduction: Giblotastoma is the most common and aggressive primary brain malignancy. Stem-like tumor cells have been shown to more closely resemble the patient tumor. However, they vary markedly regarding growth and marker expression. Therefore the aim of the following study was to carefully characterize SGLC-lines based on their in vivo self renewal potential, in vivo tumorigenicity and expression of the surrogate marker AC133.

Material and Method: Neural colony-forming cell assay (NCFFCA) was performed in a collagen matrix on 8 SGLC lines to quantify their self renewal potential. Then 10^4 cells of each cell line were implanted orthotopically into non-obese diabetic immunodeficient (NOD SCID) mice and monitored for a period of 8 weeks (short-term). In addition, for 6/8 cell lines observation period was prolonged (long-term) and for 4/8 SGLCs the implanted cell number was increased to 10^5 cells/mouse. Cytosectomies of the brains were analyzed for tumorigenicity, invasiveness, neoangiogenesis and proliferative potential.

Results and Discussion: NCFFCA revealed clonogenicity ranging from 0.13% to 18%. Concordantly, in our short-term approach 4/5 highly clonogenic cell lines developed tumors with 100% penetrance resulting in a decreased symptom-free survival. Tumor penetrance was associated with a higher AC133 content. In our long-term approach and after implanting increased cell numbers even AC133^-/- cell lines developed tumors. However, the latter were less dense and more infiltrative. In contrast, superficial implantation in the window chamber model revealed an aberrant vascular network with microvascular proliferation for all SGLC lines but was not able to delineate growth differences observed after intracranial implantation.

Conclusion: Our findings indicate a critical influence of the tumor-surrounding brain and the observation period length on tumorigenicity and invasiveness of SGLCs which needs to be taken into account when planning in vivo experiments with SGLCs.
laboratory indicated the presence of Plexin B1 protein in the nucleus in tumour cells, suggesting that plexins may have a role in the nucleus.

The aim of this study was to determine if Plexin B1 is translocated to the nucleus in prostate cancer cells.

**Material and Methods:** Immunofluorescence was done on cell lines with antibodies specific to the intracellular domain of Plexin B1. Co-localisation studies were carried out using the Velocity Software®. A cell fractionation assay was performed and cell lysates analysed by immunoblotting. A construct of the cytoplasmic domain of Plexin B1 fused with GFP was generated and its subcellular localisation in transfected cells analysed by confocal microscopy.

**Results:** Immunofluorescence of prostate cancer cell lines showed the presence of the intracellular domain of PlexinB1 receptor in the nucleus, suggesting a translocation of the protein to the nucleus. Plexin B1 was found to co-localise with DAPI staining. Further immunofluorescence studies showed that the presence of PlexinB1 in the nucleus is dependent on Semaphorin4D binding in a time-dependent manner. The translocation occurs 15min after ligand binding and decreases after 40min of ligand binding. A cell fractionation assay followed by immunoblotting confirmed the preliminary immunofluorescence data. To assess the nuclear trafficking with a functional assay, a GFP tag was attached to the intracellular domain of Plexin B1 and its translocation to the nucleus was observed in transfected cells.

**Conclusion:** In conclusion, the translocation of PlexinB1 to the nucleus suggests an interesting novel role for this receptor in the nucleus. PlexinB1 could act as a transcription co-factor and may have an impact on carcinogenesis. Further experiments are aimed at defining the nuclear localisation signal in Plexin B1 by mutating the GFP-Plexin B1 construct.

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Lung Derived Fibroblasts Influence Extracellular Matrix Composition and Dissemination of Lung Cancer Cells

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Background: There is increasing evidence of the central contribution of tumor microenvironment to tumor development and progression. The elucidation of the role of different cellular components to this mechanism might therefore provide novel insights on the transformation process and potentially indicate targets for innovative therapeutic intervention. It has been shown that extra cellular matrix (ECM) is a key regulator of neoplastic cell growth and mobility and its alterations to allow invasion, migration, and angio genesis is essential for cancer cells to move from a localized primary site.

Material and Methods: To investigate the contribution of fibroblasts to lung cancer we established primary cultures of Cancer Associated Fibroblast (CAFs N = 81) and matched Normal Fibroblasts (NFs, N = 59) from resected NSCLC and performed co-injection experiments in nude mice with A549 lung cancer cells. We also performed in vivo experiments by injecting CAFs and fibroblasts (CAFs or NFs) and in 9 tumors generated by injection of lung cancer cells alone as controls (A549S).

Results: Comparison to control tumors, heterotypic tumors displayed strongly increased levels of COL6A3 and MMP2 (p < 0.03), and reduced levels of CTSL and CTSC (p < 0.01) indicating the influence of fibroblasts on ECM composition. Moreover immunofluorescence analysis on paraffin sections of the same tumors were successfully performed to validate the results obtained by Real time PCR.

Conclusion: Taken together these data demonstrate that the process of cancer cell dissemination can be attributed in part to fibroblasts and cancer cells can dictate ECM composition, regulate dissemination of lung cancer cells and suggest that identification of factors responsible for this cross-talk has the promise of providing opportunity in devising novel therapeutic strategies.

MicroRNAs Implicated in the Regulation of NF-kappaB Activity in Glioma Tumours

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Background: Gliomas are among the most prognostically discouraging neoplasia in human and there is basically no curative option for glioma patients. Nuclear factor-xB (NF-xB) is a key effector associated with temozolomide (TMZ) resistance in glioma tumours. TNFAP3 and NKRAS2 are critical negative regulators of NF-xB. Downregulation of either of these genes is critically associated with resistance to TMZ, but the molecular mechanism of dysregulation is largely unknown. microRNAs that are implicated in the NF-xB pathway may be important regulators of resistance to alkylating agents.

Material and Methods: Glioma cells were transduced with mir-125a expression plasmid and analyzed for cell cycle arrest, apoptosis and resistance to TMZ. NF-xB activity was assessed by NF-xB-inducible reporter assays and Western blot analysis. Luciferase reporter plasmids containing mir-125a target sites were co-injected with glioblastoma cells and analyzed for cell cycle arrest, apoptosis and resistance to TMZ.

Results: For the time that TNFAP3 and NKRAS2 are directly regulated by physiological concentrations of the brain-enriched microRNA mir-125a. Consistent with these findings, overexpression of mir-125a resulted in a significant induction of NF-xB activity and, as a consequence, also in a strong upregulation of the anti-apoptotic proteins Bcl-2 and BIRC3. Glioblastoma cells overexpressing this miRNA were significantly resistant to TNF-α and TRAIL-induced apoptosis. In addition, we showed that mir-125a confers resistance to T. We are using in vivo mouse models including Pb-Cre and p63-flox, to specifically ablate p63 in the adult prostate. In parallel we are using the human non-transformed cell line RWPE1 and the human metastatic cell line PC3 to investigate a role for p63 in prostate stem cell maintenance and tumour development.

Results and Conclusion: We show for the first time that TNFAIP3 and NKRAS2 are directly regulated by physiological concentrations of the brain-enriched microRNA mir-125a. Consistent with these findings, overexpression of mir-125a resulted in a significant induction of NF-xB activity and, as a consequence, also in a strong upregulation of the anti-apoptotic proteins Bcl-2 and BIRC3. Glioblastoma cells overexpressing this miRNA were significantly resistant to TNF-α and TRAIL-induced apoptosis. In addition, we showed that mir-125a confers resistance to T. We are using in vivo mouse models including Pb-Cre and p63-flox, to specifically ablate p63 in the adult prostate. In parallel we are using the human non-transformed cell line RWPE1 and the human metastatic cell line PC3 to investigate a role for p63 in prostate stem cell maintenance and tumour development.

Results and Discussion: In wild type mouse prostate, FACS sorting, qPCR and Real time of the DCR4/510-p63 isoform in normal and tumour tissue has shown that DCR4/510 expression is highest in the normal stem cells of the mature gland. Functional studies using our in vivo mouse model with specific ablation of p63 in the adult prostate show a progressive loss of tissue structure following p63 ablation and suggest that p63 is necessary for prostate stem cell maintenance. Together, this functionally shows that p63 is a critical mediator of prostate stem cell function.
Although advanced prostate cancer is typically regarded as not expressing detectable p63 levels, we have unexpectedly found low expression levels of the p63 isoforms. Furthermore, FACS purification and analysis of the CD44/CD133-positive cancer stem cell population reveals that expression of p63 is highest in these cells.

Conclusion: Together, this work describes how Np63a is a critical mediator of normal prostate stem cell function. Surprisingly, we have also uncovered expression of p63 in a subset of cancer stem cells in prostate cancer that suggests that tumor cells may utilize the stem cell-promoting function of p63 to facilitate tumor maintenance and metastatic colonization.

[565] Phosphatidylcholine-specific Phospholipase C as a Target to Manipulate CXCR4-CXCL12 Signaling Pathway in Human Lymphoblastoid Cells

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Background: Chemokines, a family of small pro-inflammatory cytokines, and their receptors regulate a variety of immune responses. Cancer cells are known to exploit signaling through chemokine receptors for key steps of tumor initiation and progression. CXCR4, a G protein-coupled receptor, is overexpressed in cancers of different origins. The functional consequence is a sustained CXCR4 activation by the CXCL12 chemokine which regulates tumor proliferation, survival, chemotaxis and migration.

We showed that phosphatidylcholine (PC)-specific phospholipase C (PC-PLC) is able to modulate the membrane expression, and hence the signal transduction, of specific cell receptors such as CD16 in NK cells and HER2 in breast cancer cells, interfering with cell-signaling dependent functions. In this study we investigated the alterations of CXCR4 expression and PC metabolism induced by the PC-PLC inhibition in a human T-lymphoblastoid cell line (CEM).

Material and Method: Membrane localization and direct interaction of PC-PLC with CXCR4 were investigated by confocal laser scanning microscopy (CLSM), flow cytometry and immunoprecipitation techniques. The effects induced by a selective PLC inhibitor (D609) on CXCR4 membrane expression and its overall contents were evaluated in comparison with those of a well-known CXCR4 antagonist, AMD3100. MRS experiments were performed on ethanolic extracts of CEM cells, previously exposed to the D609 until 24h, using a Bruker Avance 400 spectrometer equipped with a 1H-X multilinear inverse probehead.

Results and Discussion: PC-PLC was preferentially localized on the membrane surface of CEM cells. Fine analysis of CCR4 and PC-PLC subcellular distribution demonstrated that the two proteins are physically associated and partially accumulated in lipid rafts.D609 induced a strong downmodulation of the CXCR4 receptor from the plasma membrane within 5 h. At this time point most of CXCR4 molecules internalized into early and late endosomes and a substantial amount of the receptor co-localized with lysosomes, indicating that PC-PLC desensitizes CXCR4 down-regulation as also confirmed by Western blot experiments. Under these conditions, MRS experiments provided direct evidence of PC-PLC inhibition showing an about 42% reduction of phosphocholine, direct product of PC-PLC activity (in agreement with enzyme assays) in cells exposed for 24h to D609. Moreover, PC-PLC appeared to negatively regulate the CXCR4-CXCL12 signaling pathway. Indeed, inhibition of this phospholipase inhibited inactivation of both ERK1/2 and AKT, even in the presence of the CXCR4 stimulus, this effect being higher than that observed for AMD3100 only.

Conclusion: Altogether, these data indicate that PC-PLC could play an important role in regulating the CXCR4-CXCL12 signaling pathway and suggest that, by manipulating this axis, PC-PLC may represent a potential therapeutic target for cancer treatment.

[567] N-glycan Biosynthesis Inhibitors Induce In Vitro Anticancer Activity in Colorectal Cancer Cells

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Background: During malignant transformation, changes in the expression profile of glycans can be involved in a variety of events, including the loss of cell-cell and cell-matrix adhesion, migration, invasion, and evasion of apoptosis. Besides, modulation of glycans has promising therapeutic potential for various cancer types. In this study, we investigated the in vitro anticancer activity of the N-glycan biosynthesis inhibitors (swainsonine and tunicamycin) in cells derived from colorectal cancer. We also examined whether these inhibitors are able to induce radiosensitization and toxicity when used in combination with cisplatin or irinotecan, two current anticancer drugs.

Material and Method: Three colorectal cancer cell lines were used: Caco-2, HCT-116, and HT-29. Cells were treated with drug concentrations ranging from 0.1 to 8 μM for swainsonine and 0.25 to 2 μM for tunicamycin. We examined the effects of swainsonine and tunicamycin treatment on cytotoxicity, migration, invasion, anchorage-dependent and anchorage-independent colony formation, and radiosensitivity, after 24, 48, 72, and 96 h.

Results and Discussion: Our results show that treatment with tunicamycin inhibits cellular mechanisms related to the malignant phenotype, such as anchorage-dependent and anchorage-independent colony formation, migration and invasion, in undifferentiated HCT-116 colon cancer cells, whereas swainsonine only inhibits cell migration. We also observed that tunicamycin, but not swainsonine, caused radiosensitivity in HCT-116 cells. Moreover, the combination of swainsonine with cisplatin or irinotecan enhanced their toxicity in HCT-116 cells, while the combination of tunicamycin with these drugs had no effect.

Conclusion: Given these results, we suggest that the modulation of N-glycan biosynthesis appears to be a potential therapeutic tool for colorectal cancer treatment because inhibition of this process induced anticancer activity in vitro.

[569] Anticancer Immune Responses Induced by Chemotherapeutic Agents Depends on Autophagy

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Immunogenic cell death can be obtained by using antineoplastic chemotherapies and provokes an anticancer immune response, thus increasing the efficiency of the treatment. Here, we demonstrate that autophagy, often disabled in cancer, is required for immunogenicity of chemotherapy-induced cell death. We observed that autophagy-competent, but not autophagy-deficient cancers attracts dendritic cells and T lymphocytes into the tumor bed in response to chemotherapy. Moreover, autophagy inhibition decreased the amount of adenosine triphosphate (ATP) released from dying tumor cells. This effect could be reversed by inhibiting extracellular ATP-degrading enzymes, which led to increased pericellular ATP in autophagy-deficient tumors, and thus, to the re-establishment of the recruitment of immune cells and restoration of the chemotherapeutic responses in immunocompetent hosts. Altogether, this study shows that autophagy is essential for the release of ATP from dying cells and the triggering of the immune system, and that increased extracellular ATP concentrations can enhance the efficiency of antineoplastic chemotherapies in the case of autophagy-deficient cancers.

[570] Suppression of Breast Cancer Metastasis by BMP4

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Background: The majority of deaths due to breast cancer result from the formation of metastases that arise in distant organs such as lymph nodes, lung, liver and bone. To date, there is no effective cure and current treatments are largely palliative.

Methods and Results: We have utilised a mammary tumour model comprising a series of isogenic tumours with varying metastatic capacities to reveal genetic regulators of metastasis. One of which is BMP4, a ligand in the TGF-β superfamily. The extent of metastasis in mice bearing highly metastatic 4T1.2 mammary tumours engineered to express exogenous BMP4 is markedly reduced compared to those bearing the parental 4T1.2 tumours. The inhibition of metastasis is attributable both to decreased tumour cell escape from the primary site and a marked reduction in growth of macrometastases in distant organs. BMP4 induces a number of genes in the tumour cells, including SMAD7. Enforced expression of SMAD7 can reproduce the metastasis suppression seen in BMP4 expressing tumours whilst stable knockdown of SMAD7 in BMP4 expressing 4T1.2 tumours reverses the inhibition of metastasis driven by BMP4. BMP4 also has paracrine activity leading to suppression of metastasis. Expression of BMP4 in 4T1.2 tumours reduces the mobilisation of CD11b+Gr1+ myeloid derived suppressor cells (MDSC) that have been implicated in many studies as suppressors of T cell activity and promoters of metastases. MDSC are mobilised from bone marrow by G-CSF, which is secreted by the tumour cells. BMP4 inhibits the secretion of G-CSF, thereby leading to reduced mobilisation and enhanced anti-tumour immunity. Forced expression of G-CSF in 4T1.2-BMP4 cells restored metastasis and increased their metastatic capacity. The clinical potential of BMP4 as an anti-metastatic therapy is revealed by administration of recombinant BMP4 to mice bearing 4T1.2 tumours, resulting in a significant increase in overall survival. Further, high expression of BMP4
in primary breast tumours correlates with improved disease-free survival and overall survival in a large cohort of patients.

Conclusion: Activation of the BMP4 signalling pathway is a potential therapeutic approach for patients with advanced breast cancer.

[377] Ageing and Cancer-related Gene Expression of the Human Cell Lines Transfected With K-RAS12V, BMI-1 and BCL-2 Or/and TERT M. Kamada1, T. Takahashi1, K. Aoki1, Y. Kawaiha1, T. Matsuo1, Y. Mitus1, T. Kumazaki1, Y. Kawahara1, T. Matsuo1, Y. Mitsui1, S. S90 European journal of cancer 48, suppl. 5 (2012) S25–S288 Sunday 8 July 2012 we obtained 55 cultures with normal range of lifespan (75–140 PDL). In addition, three immortal cell strains, and unexpectedly one ultra long-lived cell line (ULT-1) with life span of 166 PDL were established. IMT-1, one of immortal cell strains was confirmed to maintain long telomere length, high telomerase activity and extremely low level of p16INK4A. They showed vigorous growth even at 450 PDL. Transfomants of mutated K-RAS, BMI-1 and BCL-2 yielded higher growth rate and higher expression of BCL-2 without forming colony in soft agar gel. ULT-1, however, exhibited shortening of telomere length, high expression of p16INK4A, moderate level of Sa-b-Ga and finally reached senescence at 166 PDL with normal senescent morphology and karyotypes. Expression system of GFP and luciferase under the control of p16 promoter was developed to utilize this ULT-1 for the screening of senescence modulating agents for the study of aging and cancer attaching great importance to generation of their model cells.

Materials and Methods: K-RAS12V, BMI-1, BCL-2 and/or TERT cDNA were transfected into human lung fibroblast, TIG-1. Hygromycin B, Phleomycin D1, Puromycin or G418 were used for drug selection of their transformants. Population Doubling Level (PDL) during subcultivation was recorded. Senescence was confirmed by the decline in growth rate and appearance of senescent associated β-Galactosidase (Sa-b-Ga) staining cells. Immortality was defined as division potential with more than 200 PDL. Expressions of various genes of cell cycle, cancer specific characters, and cell death were examined by RT-PCR. Colony formation in soft agar gel was performed as a marker of malignant growth.

Results and Discussion: Of 74 individual clones of TERT transfected cells, we obtained 55 cultures with normal range of life span (<75 PDL), 16 cultures with extended life span (75–140 PDL). In addition, three immortal cell strains, and unexpectedly one ultra long-lived cell line (ULT-1) with life span of 166 PDL were established. IMT-1, one of immortal cell strains was confirmed to maintain long telomere length, high telomerase activity and extremely low level of p16INK4A. They showed vigorous growth even at 450 PDL. Transfomants of mutated K-RAS, BMI-1 and BCL-2 yielded higher growth rate and higher expression of BCL-2 without forming colony in soft agar gel. ULT-1, however, exhibited shortening of telomere length, high expression of p16INK4A, moderate level of Sa-b-Ga and finally reached senescence at 166 PDL with normal senescent morphology and karyotypes. Expression system of GFP and luciferase under the control of p16 promoter was developed to utilize this ULT-1 for the screening of senescence modulating agents for the study of aging and cancer attaching great importance to generation of their model cells.

Conclusion: ULT-1 is useful for screening senescence-modulating factors with extremely long life span, and IMT-1 or -2 from the same parent cells are useful for the study of human cancer formation from normal cells.

[378] The Subset of CD133+/CXCR4+/EpCAM− Cancer Initiating Cells is Responsible for Lung Tumor Metastatic Spreading G. Bertolini1, M. Moro1, M. Tortoreto1, R. Caserini1, U. Pastorino1, L. Roz1, G. Sozzi1,1 Fondazione IRCCS Istituto Nazionale Tumori, Department of Experimental Oncology and Molecular Medicine, Milan, Italy. 1Fondazione IRCCS Istituto Nazionale Tumori, Unit of Thoracic Surgery, Milan, Italy. Background: Late diagnosis at metastatic stage is the main reason for treatment failure of lung cancer. We previously demonstrated that the subset of CD133+ cells represents the fraction of lung cancer initiating cells (CICs) able to initiate and sustain tumor formation. Furthermore we showed that clastipan treatment enriches for a subpopulation of CD133+ lung cancer cells co-expressing CXCR4, a chemokine receptor involved in invasion and metastasis. We suggest that chemoresistant CD133+/CXCR4− subset may represent the migrating fraction of CICs involved in metastatic process.

Results: We analyzed by FACS a series of primary lung tumors (n = 30) and showed that the subpopulation of CD133+/CXCR4− cells represents 0.3% of the total population.

To test the metastatic potential, CD133+/CXCR4− and CD133+/CXCR4+ cells were sorted from patient derived xenografts (PDXs), established by direct implant of primary lung tumor tissue in immunocompromised mice, and injected intravenously in SCID mice. FACS analysis of lungs for human HLA class I and total population.

Introduction: Lung cancer is the leading cause of cancer death worldwide, and the incidence of new cases continues to rise – largely because of the dramatic increase in the number of smokers. Lung cancer is a heterogenous disease, and the different subtypes of lung cancer require different therapeutic strategies. Epithelial to mesenchymal transition (EMT) is a key event in the development of cancer metastasis. EMT is characterized by the acquisition of stem-like properties (i.e. increase of CD133+ cells fraction, increased expression of stemness genes and enhanced tumorigenicity in vivo). Moreover we observed that EMT process could generate a subset of migrating CD133+/CXCR4 lung CICs that do not express the epithelial marker EpCAM.

Using PDX models we observed that tumor cells colonizing mice lungs were enriched for CD133+/CXCR4+/EpCAM− subset and expressed high levels of stemness genes and EMT-related genes compared to bulk tumor cells, suggesting that disseminating lung tumor cells are endowed with stem like features and mesenchymal traits.

In this case, wild-type KRAS cells were affected by PI3K inhibition, resulting in cells with well-characterised selective advantages, but also less well understood vulnerabilities.

Materials and Methods: In order to affect tumour-specific vulnerabilities associated with the oncogenic state, we used a panel of twenty-six non-small cell lung cancer (NSCLC) cell lines, half of which carry an activating KRAS mutation, to examine the activity of small molecule inhibitors targeting pathways directly controlled by RAS, such as RAF/RAF1/MEK or PI3K/AKT/mTOR, as well as drugs directed against other targets (such as HSP90, topoisomerases or the proteasome).

Results: Knock-down of KRAS using siRNA indicates that KRAS mutant cells require KRAS expression to maintain viability. Moreover, KRAS depletion produces a clear inhibition of ERK and AKT signaling. Viability assays showed that cells harbouring an activated KRAS oncogene were more sensitive to MEK, RAF and IGFR-1 inhibitors than cells carrying only wild-type KRAS alleles. In contrast, no differential loss of cell viability was observed when cells were treated with either PI3K or AKT inhibitors. Combining inhibitors of IGFR-1 and PI3K/AKT in a synergistic and pro-apoptotic manner resulted in KRAS mutant cell lines. Similar effects were observed upon simultaneous treatment with PI3K and MEK inhibitors. However, in this case, wild-type KRAS cells were also affected by PI3K inhibition.
resulting in a less-pronounced differential effect. Pathway inhibition analysis showed that, whereas MEK and PI3K inhibitors respectively decreased ERK and AKT phosphorylation in all cell lines, IGF-IR inhibitors decreased AKT phosphorylation only in KRAS mutant cell lines. Inhibition of AKT phosphorylation correlates with effectiveness of IGF-1R inhibitor in blocking cell proliferation.

**Conclusions:** Our results indicate that mutant KRAS activation of PI3K requires coordinate input from IGF-1R. These findings suggest potential therapeutic strategies for NSCLC tumours harbouring KRAS mutations.

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**Expression Profile of SETD Methyltransferase Family Genes in Breast Cancer Cell Lines**

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**Background:** Lysine-methyltransferases are key enzymes that post-translationally modulate gene expression in histones and play a key role in gene expression, epigenetic regulation, and as determinants of survival in malignant cells. The involvement of epigenetic modifying enzymes in carcinogenesis has gained a great deal of attention in the past few years. However, little is known about the role of SETD methyltransferase family genes in this process. In this study we investigate the expression of all SETD family genes in a panel of 7 breast cancer cell lines and a non-tumor counterpart.

**Material and Methods:** We selected seven breast cancer cell lines (MCF7, CAMA1, SKBR3, MDA-MB-231, MDA-MB-436, MDA-MB-468 and HCC1954) and a matching cell line from normal B lymphoblastoid (HCC1954-BL) for the expression analysis. Total RNA was isolated and cDNAs were synthesized. Quantification of 9 SETD family genes (SETD1A, SETD1B, SETD2, SETD4, SETD5, SETD6, SETD7 and SETD8) was performed by semi-quantitative PCR and Real Time PCR (qPCR). All PCR were normalized with b-actin. Results were analyzed by the comparative 2^−ΔCt method and each cell line was compared with the control group of normal cells HCC1954-BL.

**Results:** In accordance to previous reports, we found SETD7 to be overexpressed in all 7 cell lines. SETD6 is highly expressed in all cell lines, except in MCF7 and MDA-MB-436, compared to the normal cells. SETD8 was also found to be highly expressed in HCC1954-BL, MCF7, MDA-MB-231 and MDA-MB-436. All together, this data points for a possible role of SETD6 and SETD8 in breast carcinogenesis. However, further studies are necessary. Interestingly, SETD4 was specifically highly expressed in the ER (+) breast cancer cell lines HCC1954, MCF7, MDA-MB-231 and MDA-MB-436, but not significantly found in ER (-) cells such as MCF7. This suggests SETD4 expression as a possible specific molecular marker. We found SETD1B to be 4 fold more expressed in 3 cell lines, including HCC1954, in comparison to the matching normal line. SETD1A was also more expressed in HCC1954 and MDA-MB-436 cells. The role of these two genes in carcinogenesis has also not been fully elucidated. The genes SETD2, SETD3 and SETD5 were not significantly expressed in the majority of breast cancer cell lines. Their expression was only found to be significant in HCC1954 in comparison to its matching normal cell line. SETD2 was recently reported as a tumor suppressor, being significantly less expressed in breast cancer and in later tumor stages. The result obtained in this study may reflect the characteristic of the primary stage IIA cell line HCC1954.

**Conclusions:** The comparative analysis of SETD family gene expression in cancer cell lines provides new insights on the relation between SETD genes and tumorigenesis. These findings can serve as platform for further analysis in clinical samples from patients, aiming to point new potential molecular markers or targets for therapeutic intervention in breast cancer.

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**Apoptotic Intrinsic Pathway as an Essential Key for the Human Salivary Glands (HSG) Branching Morphogenesis**

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**Introduction:** Since the disclosure of apoptosis and its relation to tissue development, homeostasis and pathogenesis, the mechanisms supposedly involved in this process have been investigated. Then, it has been well established that apoptosis occurs by two major pathways, intrinsic and extrinsic, sometimes showing a cross-reaction. Bcl-2 family proteins can control the intrinsic pathway through the balance between their pro and anti-apoptotic factors. Considering apoptosis as an essential key for the development of many tissues, this study purposes to illustrate the presence of apoptosis and its regulation at earlier phases of salivary gland morphogenesis.

**Material and Methods:** Immunohistochemical reactions for Bax, Bak, Bax-Bak, Bid, Bcl-XL and Bcl-X were performed on breast cancer cell lines from normal cells and some other tissues were employed as controls. Results and Discussion: Bax, Bak and Bcl-XL were significantly expressed during almost all stages of salivary gland development and Bcl-X. Bad and Bid showed only a focal positivity, being present in ductal and epithelial (ducts) cells, both in nucleus and cytoplasm. Mature HSG were employed as controls and were negative as well as Bcl-2 and acinar cells from fetuses. Bax and Bcl-XL were more abundant at earlier phases of salivary gland morphogenesis. In the luminal space, whilst the nuclear expression of Bcl-XL was more concentrated at latter stages. Regarding that the intrinsic pathway depends on the balance between Bcl-2 family protein and anti-apoptotic factors, those results were consistent, once Bcl-XL seems to control the apoptotic cell fate after tumor formation. Besides, evidences indicate a mutual regulation between Bcl-2 and Bcl-XL, being a possible reason for the absence of Bcl-2. Bad in turn acts binding to anti-apoptotic factors, Bcl-XL is an isoform of Bcl-X and Bid transmits death signals from the extrinsic pathway to the intrinsic. Thus, their almost negative expression indicates a secondary role during the human salivary gland morphogenesis, reinforcing the importance of the intrinsic apoptotic pathway during this process.

**Conclusion:** Apoptosis seems to have an important role in the formation of the ductal network of HSG mainly through the intrinsic pathway. Associated with other proteins, Bcl-2 family members can contribute for the gland development as well as for the acquisition of new data.

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**Extra-cellular Matrix Stiffness and Immune Cells Infiltrate Are Associated With Breast Tumor Phenotype**

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**Introduction:** Local tumor progression is influenced by the dynamic interplay between the genetically-modified epithelium and the associated microenvironment. Data indicate that the extra-cellular matrix (ECM) stiffness progressively as mammary tumors evolve and enhancing ECM stiffness promotes mammary tumorigenesis while inhibiting ECM stiffening reduces tumor progression (Butcher et al., 2009; Levental et al., 2009). In addition, tumor progression is associated with altered inflammatory infiltrate (Ruffell at al., 2011), which can influence ECM remodeling. This poses the interesting possibility that inflammation could regulate tumor progression by inducing ECM stiffness. Accordingly, we hypothesize that immune infiltration and ECM stiffness may be integrated into a tumor histology phenotype associated to breast cancer progression and ECM stiffening.

**Material and Methods:** We obtained breast tissue with benign and invasive ER positive and negative cancer from treated and non-treated patients. Tissues were subjected to immune profiling, IHC histological and immunocytokinetic analysis (H&E; 3DFFAK; YAP-TAZ; LOX; FN) and biomechanical assessment (Atomic Force Microscopy; Structured Illumination Polarized Imaging, Two Photon Imaging).

**Results and Discussion:** Preliminary data show a significant increase in ECM stiffness in tumor tissue. The highest stiffness is located at the tumor edge (2-4 fold greater). Intriguingly, we observed that ER negative tumors are stiffer than ER positive tumors. ECM stiffness correlates with infiltrated parallel collagen fibers and ECM birefringence. Moreover, ECM stiffness is lower in treated tumors (40% lower) with the most striking reduction in ECM stiffness in ER negative samples. Our preliminary data suggest that ECM stiffening may be correlated with the percentage of macrophages in the immune infiltrate.

**Conclusions:** (1) tumor progression is associated with marked fibrosis, accompanied by collagen deposition and linearization and an increase in ECM stiffness; (2) tumor progression is associated with high tissue stiffness; and (3) ECM stiffening and macrophage infiltration level may be correlated with ER/PR status. This data should lead to a deeper understanding of breast cancer microenvironment and its role in tumor response to therapy.

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**Transformation and Aging of Human iPS Cell Teratoma-derived Cells**

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**Background:** Generation of human cancer cell lines from normal cells is hardly successful without oncovirus infection. Tumorigenicity of induced pluripotent stem cell (iPSC) is considered as a risk factor of their transplant. We aimed to generate transformed human cells from human iPSC teratoma.
Material and Methods: Human iPSC were established from normal lung fibroblast, by transfecting Yamanaka factors and injected into severe combined immunodeficient (SCID) mice. Cells were isolated from each teratomas at different stages by enzymatic digestion. Genotype was examined by real-time PCR. Aging was confirmed by senescence-associated β-galactosidase staining and colony formation. Transformation was examined by colony formation in soft agar gel and tumor formation in SCID mouse.

Results: Twelve clones of human iPSC from TIG-1 formed human teratomas. Histological analysis showed the existence of glandular epithelium, melanocyte, bone, tooth, retinal tissue, cartilage-like tissue, striated muscle, hepatocyte like cells and connected tissue. The isolated cells from the teratomas were cultured in vitro exhibiting various types of cells with distinct morphology. Most of the cells ceased to divide sooner or later. Cell aging was confirmed by SA-β-Gal staining. Colonies of clonal growth were isolated for further analysis of differentiated state showing expression of endothelin, CD34 and fibroblast specific protein etc. Interestingly, however, from one iPSC teratoma case, we could isolate big colonies forming in soft agar gel cultures. The isolated colony from the gel grew rapidly and long-term subcultivation was succeeded without senescent phenotypes. Some oncogenes but not iPSC marker genes were expressed in this cell line. Tumorogenic activity in SCID mouse was underway.

Conclusions: A transformed cell line was established from human normal cell via its iPSC teratoma, though most of cells from teratoma exhibited cellular aging.

Functional properties of PPFIA1, Located at the 11q13 Amplification Region, in Epithelial Cancer Cells

H. Pehkonen1, T. Lepikhova1, R. Louhimo1, N. Peitsaro1, M. Imai1, S.92 European Journal of Cancer 48, suppl. 5 (2012) S25–S288 Sunday 8

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ERα using an estrogen-like compound targets and causes apoptosis in castrate resistant prostate stem cell populations.

Materials and Method: Castrate mouse models were treated with estrogen receptor beta selective agonists to show cellular mechanism of action. Human and mouse specimens were used to show direct action on the FACs sorted stem cell populations.

Results and Discussion: Compared to castration and androgen deprivation, apoptosis due to selective activation of estrogen receptor beta (ERβ) is mechanistically different. The cellular targets are also different and ERβ agonist significantly stimulates apoptosis in murine basal cells that are unaffected by castration. The regenerative potential of agonist treated tissue is reduced and after two cycles of this treatment, cystic atrophy occurs due to successive depletion of p53+ basal cells indicating that the stem/progenitor cells are responsive to ERβ agonists. As clonogenicity and self-renewal of murine prostatic stem/progenitor cells were attenuated by agonist treatment, it was predicted and shown that both murine (Lin−Scal1+CD49fhi) and human (CD49fhiTrop2hi) prostatic basal stem cells are direct targets. In combination, selective stimulation of ERβ has combined added benefit to androgen-deprivation, causing apoptosis of basal, luminal and intermediate cells in quiescent post-castration tissues.

Conclusions: Our results reveal a novel benefit and cellular mechanism of ERβ activation, and suggest that combining selective activation of ERα with androgen-deprivation is a feasible therapeutic strategy to target regenerative stem cells proven to be cells of origin of prostate cancer.

References:

Mechanisms of Tumor Malignization After Anti-angiogenic Therapies in Renal Cell Carcinoma
L Moserie1, M. Martinez2, G. Jiménez1, A. Vidal2, O. Casanovas1.

Introduction: Anti-angiogenic therapy has been shown to significantly reduce or stop tumor growth. Nevertheless these therapies fail to produce durable effects and many drugs have been developed to counteract this process, thus impairing tumor development. Nevertheless these therapies fail to produce durable effects and to significantly modify the patients’ long-term survival due to tumor adaptation and subsequent resistance to therapy. This is the case of renal cell carcinoma (RCC) patients that are currently treated with VEGF signaling inhibitors. Furthermore, recent findings in animal models have documented that anti-angiogenic therapies switch on an invasive and metastatic phenotype as adaptation to the inhibition of VEGF pathway.

Materials and Methods: We have developed several RCC tumor graft mouse models based on orthotopic implantation of human RCC tumors derived from primary biopsies. By defining different parameters of local tumor invasion, we have evaluated the effects of VEGF signaling inhibition in the invasive behaviour of the RCC tumors. Furthermore, molecular and immunohistochemical characterization is ongoing to find out possible driving forces of increased tumor invasiveness and evaluate the impact of anti-angiogenic therapy on the tumor’s biological features.

Results: Our preliminary results showed that the inhibition of the VEGF pathway exacerbates tumor invasiveness as suggested by the more complex shape of the invasive front and the higher width of invasion into normal kidney tissue of treated tumors. Moreover, the molecular and immunohistochemical characterization of the tumor in vivo demonstrates that anti-angiogenic therapy induces a signal rewiring that could allow for increased capacities of invasion, dissemination and metastasis.

Conclusions: The understanding of the mechanisms related to the tumor reaction to anti-angiogenic therapy represents the basis for the development of enduring anti-tumor treatments. Additionally, the identification of molecular signature driving tumor malignization could be used as a predictive factor of response to current anti-angiogenics and to identify new therapeutic targets to improve their efficacy and avoiding the possible negative side effects of these treatments.
Our in vitro assays reveal the effect of various exosomes on OC spheroid attachment to mesothelial cells and on their clearance. Mice, grafted with OC exosomes, showed more aggressive tumor spread and shorter survival.

Conclusions: To our knowledge, this is the first study to evaluate the differential expression of miRNAs in exosomes derived from OC ascites fluid. We believe that the differential expression and the distinct signatures of miRNAs possess diagnostic, prognostic and therapeutic potentials.

We also show that OC effusion derived exosomes might have autocrine or paracrine effects on cells, thus enhancing the oncogenic potential of cancer cells, or sensitizing the body to malignant dissemination.

**Photodynamic Therapy Mediated by Hypericin, a Natural Endoplasmic Reticulum-localized Drug, Activates Autophagic Pathways That Increase the Resistance to the Therapy**

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**Background:** Photodynamic therapy (PDT) is an emerging therapeutic modality used in the treatment of neoplastic and non-malignant diseases, where a combination of a light-absorbing drug or photosensitizer, molecular oxygen and light generate high concentrations of reactive oxygen species that kill tumor cells by overwhelming their antioxidant defenses. In previous works, we showed that PDT sensitized the endoplasmic reticulum-localized photosensitizer hypericin (Hyp-PDT) induces a rapid drop in sarko(endoplasmic)-reticulum Ca2⁺-ATPase (SERCA) pump protein levels, loss of Ca2⁺ homeostasis, ER stress and mitochondrial apoptosis. It also activates (macro)autophagy and the more selective chaperone-mediated autophagy (CMA) which increase the resistance against PDT. A recent transcriptome-analysis indicated that the autophagic adaptor p62/SQSTM1 (sequestosome 1) mRNA is significantly up-regulated after Hyp-PDT in a p38α-dependent way. In this work, we unravel the role of the autophagic adaptors p62/SQSTM1 and NBR1 (neighbour of Brc1 gene) in triggering the formation of ROS-induced protein aggregates to the autophagy machinery after Hyp-PDT and the role of p38α and Nf2/ARE pathways in the coordination of this process.

**Material and Method:** Cell lines: p38α+/− and p38α−/− transformed MEFs, p62−/− and p62−/−-transformed MEFs, HeLa and T24 cells. Protein lysates were fractionated for protein detection by Western Blot. Immunocytochemistry techniques were used to visualize activation of autophagy. siRNAs were used to downregulate gene expression.

**Results and Discussion:** p62 levels accumulated in the protein-aggregates fraction after Hyp-PDT in a time-dependent manner which correlated with a continuous accumulation of ubiquitinated proteins. Immunocytochemistry analysis confirmed that these aggregates co-localize with GFP-labeled LC3, an autophagosomal marker. Protein analysis on p38α+/− and p38α−/− and on T24 cells in presence of the transcription inhibitor actinomycin D confirmed the p38α- and Nf2/ARE-dependent process of this.

**Conclusion:** Hyp-PDT stimulates the formation of p62-containing ubiquitinated protein aggregates which interact with LC3 and are degraded through autophagy. This p62 up-regulation is transcription, p38α-/− dependent. Since activation of autophagy is a resistance mechanism to PDT, understanding its activation may help to ensure the complete success of the treatment.

**Role of Glioblastoma Cell Metabolism in Response to Anti-RTK Therapy**

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Glioblastomas (GBMs) are among the most human lethal tumors, despite the progress in surgery, radiotherapy and chemotherapy. Recent advances in the understanding of brain tumor biology have identified some promising therapeutic targets, such as receptor tyrosine kinases (RTKs). Actualy, several clinical trials are ongoing to determine the efficacy of such RTK-specific drugs in GBM cells, such as the kit and PDGFRα inhibitor, imatinib. However, previous results from our group have shown that imatinib increases cellular invasion in GBM in vitro models. And, lactate release by cancer cells is one of the mechanisms associated with increased tumor invasion. Therefore, we analyzed GBM cell metabolism in response to imatinib.

Using the SNB-19 and U251 GBM cells we observed that imatinib treatment induced an increase in glucose uptake and lactate release into the extracellular medium. In order to understand whether increased glycolysis was related to imatinib resistance, we performed a combined treatment using imatinib and glycolytic interfering agents, lonidamine, an inhibitor of glycolysis and sodium azide that increases the rate of glycolysis. By SRB assay, we observed that treatment with sodium azide led to an increase in the IC50 of imatinib, whereas no effect was seen with lonidamine. Glucose uptake and lactate release quantification revealed that glioblastoma cells exposed to sodium azide showed a higher glucose uptake and lactate release, whereas the opposite was verified with lonidamine.

In conclusion, this is the first study that analyses the role of cellular metabolism in response to imatinib in glioblastoma cells. Our results suggest that sodium azide cannot be used as a combinatorial drugs to increase the efficient of imatinib, since this increases glycolysis in glioblastoma cells. Additionally, lonidamine, which was competent in the reduction of glycolysis, did not provide an improvement in the response of glioblastoma cells to imatinib. Further studies are needed to understand the impact of imatinib in GBM glycolysis and how to explore that in a therapeutic way.
**Material and Methods:** Recombinant adenoviral vectors with RGD-modified fiber (AdRGD) and a p53-responsive promoter (PG) were constructed containing a recombinant adenovirus (rAdRGD) or the combination of both. Evaluation of in vitro antiproliferative effect of transgenes in B16F10 cells (B16, mouse melanoma, p53 wt) was done by annexin/PI staining and MTT assays. Bystander effect was evaluated by cell cycle analysis of populations transduced with different proportions of the viruses. Antitumor effect in vivo was observed by treatment of established LLC1 tumors (mouse lung carcinoma, p53 wt) with intratumoral injection of AdRGD in C57BL/6 mice. Involvement of immune response was evaluated by second tumor challenge at contralateral flank of mice with a developed and treated first tumor.

**Results and Discussion:** Cell death was resulted from the p19Arf and IFN transduced mixture (74% subG0), yet single gene transfer yielded only half the number of subG0 cells. A similar result was seen by measurement of cell viability with MTT. Evidence on a bystander effect was revealed when approximately 50% subG0 cells were observed, even though only 10% of the cells had been transduced with IFN. In a population of cells transduced with p19Arf, when 10% of them also expressed IFN, the number of subG0 cells increased to 68%, compared to transduction of p19Arf alone, which results in 45% subG0 cells. This indicates that p19Arf can sensitize cells to death by IFN bystander effect. In vivo assays with the LLC1 model have shown that in situ gene therapy of p19Arf and IFN combination was more effective to inhibit tumor progression and increase survival than application of a single gene. These animals were then challenged with the implantation of a second tumor, revealing greater retardation of growth at the secondary tumor site in mice treated with the combined gene therapy at the primary tumor locus as compared to animals that received single gene treatment.

**Conclusion:** The use of p53-responsive vectors to express p19Arf and IFN represents a potential strategy for melanoma and lung carcinoma tumor suppression. We have shown that complementation of the p53/Arf and interferon pathways in the primary tumor may generate a strong bystander effect as well as immune stimulation.

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**Aspartame Induces Angiogenesis in Chick Chorioallantoic Membrane**

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**Background:** Angiogenesis is the process of generating new blood vessels from preexisting vessels and is considered one of the key pathological conditions. Aspartame is the most widely used artificial sweetener and is added to a wide variety of foods, beverages, drugs, and hygiene products.

**Material and Methods:** The purpose of the present study was to evaluate the effect of aspartame in chick chorioallantoic membrane angiogenesis model in vivo.

**Results:** In this well characterized model, aspartame induces angiogenesis in a concentration-dependent manner. Compared with the normal group, aspartame group has significant increased vessels proliferation.

**Conclusions:** These results provide evidence that aspartame induces angiogenesis and may be important for angiogenesis-dependent human diseases.

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**Induced Expression of Sox2 Inhibits Glioma Progression in Vivo by Stimulating Ablentiment Differentiation**

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**Background:** Sox2 is a transcription factor which is expressed in neural stem cells during the development of the brain. Sox2 is known to keep the neural stem cells immature and in a proliferating state. The counteracting partner of Sox2, Sox21, is often co-expressed with Sox2 but is suggested to induce neural differentiation. In this study we have investigated the relationship of Sox2 and Sox21 in glioma, is it possible to inhibit glioma progression or growth by inducing the expression of Sox21?

**Material and Methods:** To regulate the expression of Sox2/Sox21, we have used an inducible cell system (tet-on), by which we can turn on the Sox21 expression in glioma cells. We have injected Sox21-inducible glioma cells subcutaneously into the flank as well as orthotopically into the brain of SCID mice.

**Results:** We have shown that both Sox2 and Sox21 are expressed in all tested human brain tumors both in children and in adult. Further, we have found a correlation between the expression of Sox2, GFAP and Sox21 and the lack of Fibronectin (FN) in glioma cells and we have suggested that glioma comprise of at least two different cell populations: Sox2/GFAP/Sox21/FN and Sox2/GFAP/Sox21/FN. Increased expression of Sox21 in Sox2/GFAP/Sox21/FN glioma cells in vitro showed a downregulation of glioma progression, which resulted in decreased cell proliferation and induced apoptosis. When injecting the Sox21-inducible glioma cells in vivo they developed high-grade glioma-like tumors. In addition, when the glioma cells were stimulated to express Sox21 the developing tumors were significantly smaller and the tumor free survival was increased extensively. Tumors with an increased expression of Sox21 had a lower expression of Sox2 and a prominent induction of S100 as well as a modest increase of Tuj1 and CNPase. Consequently, induced expression of Sox21 appears to inhibit or delay glioma progression and further reduce the cancer stem-like properties of the Sox2/GFAP/Sox21/FN glioma cell and induce differentiation in vivo. It has been suggested that Sox2 and Sox21 is expressed in balance during the development and our studies indicate that this balance is disturbed during gliogenesis.

**Conclusions:** Sox2 seems to be expressed in all types of human brain tumors which support the idea that Sox2 is involved in brain tumor initiation and/or progression. Further, the finding that an induction of its counteracting partner Sox21 in glioma cells increased the tumor free survival is very encouraging and makes Sox2 or down- or upstream effectors such as Sox21 interesting targets for novel therapy of brain tumors.

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**Generation of Diverse Transformed Human Cell Lines Via HiPS Teratoma**

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**Introduction:** Most of the cancer cell models have different genetic backgrounds and may not sufficiently mimic normal cell to cell communication to understand the mechanisms of cancer formation. iPSCs are pluripotent and forms teratoma in vivo. Thus, we challenged for generation of diverse malignant tumor cell lines from human iPS teratoma-derived cells.

**Material and Methods:** iPSCs were established from normal human lung fibroblasts by transfecting c-MYC, KLF-4, SOX-2 and OCT3/4. Teratomas formed in SCID mice with iPSC injection were histologically analyzed and digested to single cells for culture. Subcultivation was performed in DMEM
medium with 10% FBS. Gene expression was examined by RT-PCR. Malignancy was checked by colony formation in soft agar gel.

Results and Discussion: Among 22 cases of PSCoHo-tumors, we confirmed 13 teratomas showing various differentiatied histology such as blood vessel, granular epithelium, melanocyte, retinal tissue, cartilage-like tissue, striated muscle. Primary cultured cells exhibited various types of morphology, growth properties and some of these colonies were cloned keeping distinct properties, though most of them ceased to divide eventually. Colony formation, however, in soft agar gel by primary cultured cells (passage 0) of K12-teratomaderived cells, but not other iPSC-teratomas, enabled us to isolate various transformed cell lines. Among them, clone1 had small round or spindle-like shape, clone 3 fibroblastic, clone 6 epitheloid, clone 8 endothelial like. Their growth properties were distinguishable each other. Gene expression pattern of TERT, CD31, NANG confirmed distinctive phenotypes of the cell lines.

Conclusion: Diverse transformed human cell lines with identical background were successfully generated via hiPS-teratoma.

The Role of Sphingosine Kinase Isoforms and Their Receptors in Glioblastoma Multiforme

K. Quint1, M. Kolodziej2, H. Strik3, N. Stiel1, M. Ocker1

Material and Methods: The expression of SK1 in 13 teratomas from primary, secondary and recurrent glioblastoma patients. These data warrant further studies to understand the distinct roles of SK1, 2 and S1PR1−5 in primary, secondary and recurrent glioblastoma. The S1P analogue FTY720 is has potent antiproliferative effects in the investigated cell lines and could prove to be a potent drug in treating glioblastoma.

Conclusion: The Sphingosine pathway is altered in glioma and its components show distinct expression patterns in the investigated histopathological entities. The S1P analogue FTY720 is has potent antiproliferative effects in the investigated cell lines and could prove to be a drug in treating glioblastoma patients. These data warrant further studies to understand the distinct roles of SK1, 2 and S1PR1−5 in primary, secondary and recurrent glioblastoma.

Non-invasive Measurement of Tumour Interstitial Fluid Pressure Using Magnetic Resonance Imaging

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Introduction: Tumours exhibit raised interstitial fluid pressure (IFP), which has been shown in tumour xenograft models to be heterogeneously distributed, with high pressure in the centre and lower pressure towards the periphery [1]. This raised pressure impacts on drug delivery [2], is implicated in the development of metastasis [3] and is generated by the oxygen gradient from the tumour. ConvectionMRI provides a quantitative measurement of the oxygen gradient, which is used to accurately quantify IFP. We have used this technique to investigate the spatial relationship IFP and vascular perfusion, non-invasively during tumour growth and in response to therapy with the vascular disrupting agent ZYBRESTAT™ (CA4P).

Materials and Methods: Experiment 1: MF1 nu/nu mice were injected subcutaneously with 5×106 LS174T (n = 6) colorectal carcinoma cells. Tumours were scanned at day 16 using a 9.4T Agilent VNMRS scanner. ConvectionMRI provided an estimate of interstitial fluid velocity (IFV), from which IFP was estimated using a fluid mechanical model. These measurements were compared with direct measurements of IFP using a clinical pressure transducer. Experiment 2: At 10 days following inoculation, a second group of mice were scanned every three or four days. IFP and blood flow were measured using convectionMRI and arterial spin labelling (ASL) MRI, respectively. At 21 days following inoculation, mice were treated with 100mg/kg of ZYBRESTAT™ and scanned 24 hours later. Changes in blood flow and IFP were assessed during tumour growth and at 24 hours following therapy.

Results and Discussion: The spatial patterning of interstitial velocity maps revealed radial convection patterns from the centre of tumours. Measurements of IFP from these data were significantly correlated with measurements using a pressure transducer, with an average central IFP of 14.5 ± 7.2 mmHg. During tumour growth, blood flow decreased with tumour volume whilst IFP increased. Following therapy, blood flow significantly decreased and IFP significantly increased (p < 0.01, paired t-test). This response is consistent with the increase in vascular permeability known to be induced by ZYBRESTAT™ [4].

ConvectionMRI, a novel technique presented here, was shown to provide accurate, non-invasive measurements of tumour IFP. Furthermore, this technique, which shows good potential for translation into the clinic, can provide a quantitative measurement of response to therapy and help elucidate aspects of tumour pathophysiology, such as the mechanisms underlying the development of high IFP in tumours.

Reference(s)

Evaluation of MicroRNA Secretion From Cancer Cells in Vitro

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Introduction: MicroRNAs (miRNAs) are small non-coding single stranded RNA molecules, which are typically 19–25 nucleotides in length. miRNAs have been discovered to play a central role in cancer development and progression. Originally, miRNA profiling was performed on tumour tissue. More recently, miRNAs have been found to be present in the circulation of cancer patients. This has raised their potential as circulating biomarkers of disease. However, the specific source and role of miRNAs in the circulation is unknown and requires elucidation to determine their true potential as novel biomarkers of disease.

Aim: The purpose of this study was to determine the optimal method for isolation of miRNAs secreted by breast cancer cell lines and patient tumour tissue explants in vitro.

Materials and Methods: Three breast cancer cell lines were employed: T47D, MDA-MB-231, and SK-BR-3. Tissue explants were harvested in theatre with informed patient consent, and included tumour, tumour associated normal and diseased lymph node samples. Following 24hr incubation of cells or explants in medium, conditioned media containing all factors secreted by the cells were harvested, centrifuged at 1,000 rpm to remove cellular debris, and stored at −20°C until required for miRNA extraction. MiRNAs were also extracted from cells for comparison. Each sample underwent Trizol® phase separation followed by extraction using three commercially available miRNA isolation kits: miReagent® mini (Qiagen), miVaNA® (Ambion) or RNACeous® (Ambion).

Results and Discussion: MiRNAs were detected in all media samples collected from cell lines and fresh tissue explants. However, there was remarkable variation in yield depending on the extraction method used. Aliquots of the same samples were extracted using the three methods, with miVaNA® consistently demonstrating the strongest results, in terms of quantity and yield (Table 1). A range of miRNAs in these samples were successfully amplified by RT-qPCR.

Table 1. Comparison of extraction methods for miRNA isolation from cell conditioned media

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total miRNA yield (ng)</th>
</tr>
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<tbody>
<tr>
<td>T47D</td>
<td>6320</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>3152</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>6570</td>
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MiRNA yield from cell conditioned media from tissue explants also contained significant levels of miRNA. As expected, due to the high cellular content, yields were higher from...
**CREB Regulates the Autocrine Induction of TGF-β in Glioblastoma**

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Glioma is the most common primary tumor of the brain, and glioblastoma (GBM, a grade-IV glioma) is one of the most aggressive types of brain tumors. TGF-β is highly active in GBM, and elevated TGF-β activity confers poor prognosis. Aggressive tumors tend to acquire high TGF-β activity through diverse mechanisms including the overexpression of the TGF-β ligand by tumor cells or tumor stroma cells. In this work, we observed that in tumor cells TGF-β induces its own expression generating a malignant autocrine loop. This phenomenon explains the increased secretion of TGF-β and hence the hypervascularization of the TGF-β pathway present in GBM. We identified CREB as the transcription factor that mediates TGF-β autocrine loop. CREB is activated by the PI3K and p90RSK signaling pathways. Activation of PI3K or p90RSK could trigger CREB to mediate TGF-β expression. This work provides new molecular targets to restore normal TGF-β function as well as new therapeutic strategies against this disease.

**Panaxydol Induces Apoptosis Through an Increased Intracellular Calcium Level, Activation of JNK and P38 MAPK and NADPH Oxidase-dependent Generation of Reactive Oxygen Species**

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**Background:** The root of Panax ginseng has long been used in the traditional medicine in far eastern countries such as China, Korea and Japan for increasing stamina and resistance to stress and promoting longevity. Panaxydol is a C17 polyacetylenic compound derived from the root of Panax ginseng (Korean ginseng), and has been reported to induce cell cycle arrest and apoptosis. In this study, we showed that panaxydol triggered apoptosis preferentially in transformed cells and investigated the signaling mechanisms.

**Materials and Methods:** Apoptosis was measured by Annexin-V and PI staining. Generation of reactive oxygen species was monitored by H2DCF-DA staining. Change in cytosolic and mitochondrial [Ca2+] was measured by fura-2 and rhod-2 staining, respectively.

**Results:** Panaxydol induced apoptosis preferentially in transformed cells with a minimal effect on non-transformed cells. Furthermore, panaxydol was shown to induce apoptosis through an increase in intracellular [Ca2+] ([Ca2+]i), activation of JNK and p38 MAPK, and generation of reactive oxygen species (ROS) initially by NADPH oxidase and then by mitochondria. Panaxydol-induced apoptosis was caspase-dependent and occurred through a mitochondrial pathway. ROS generation by NADPH oxidase was critical for panaxydol-induced apoptosis. Mitochondrial ROS production was also required, however, it appeared to be secondary to the ROS generation by NADPH oxidase. Cytosolic ROS induced ER stress which then triggered mitochondrial [Ca2+] increase and subsequently they ROS production. Activation of NADPH oxidase was demonstrated by the membrane translocation of regulatory p47phox and p67phox subunits and shown to be necessary for ROS generation by panaxydol treatment. Panaxydol triggered a rapid and sustained increase of [Ca2+]i, which resulted in activation of JNK and p38 MAPK. JNK and p38 MAPK play a key role in activation of NADPH oxidase, since inhibition of their expression or activity abrogated membrane translocation of p47phox and p67phox subunits and ROS generation.

**Conclusion:** These data indicate that panaxydol induces apoptosis preferentially in cancer cells, and the signaling mechanism involves a [Ca2+]i increase, JNK and p38 MAPK activation ROS generation through NADPH oxidase and ER stress.

**Synthetic Cleanane Triterpenoids, HIXOMOL and Br-HIMOLID, Induce Autophagy in Breast Cancer Cells Through Transcription Factors and Beclin 1-dependent Mechanism**

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**Introduction:** Triterpenoids such as oleanolic acid are known for their cytotoxic effects on cancer cells in vitro. However, activity of this natural compound is associated with different side effects and non selective mechanism of action. Thus, its modifications in order to achieve safe and effective anticancer agents that could significantly reduce disease-related morbidity and mortality and improve prognosis are desirable. Here we present new synthesized derivatives of oleanolic acid, HIXOMOL and Br-HIMOLID and their activity against two breast cancer cell lines.

**Material and Method:** MCF7 (ER+) and MDA-MB-231 (ER−) breast cancer cells were treated by methyl 3-hydroxyiminio-11-oxolean-12-en-28-oic acid (HIXOMOL) and 12α-hydroxy-3-hydroxyiminoolean-28→13-olide (Br-HIMOLID) in different concentrations (up to 50 μM) in vitro. After incubation for 24 h, cell viability was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. Autophagy was identified by confocal microscopy and western blot (WB) followed by quantification of the autolysosome-associated LC3 protein. To asses the mechanism of studied compounds action, WB analysis of Beclin 1 and transcription factors i.e. p53 and NF-κB was performed.

**Results and Discussion:** We found that HIXOMOL and Br-HIMOLID treatment in non-cytotoxic concentrations led to an induction of autophagic-programmed cell death. Observed activity of the compounds was dependent on cell characteristics. In MCF7 cells the autophagy process was expressed more intensively after HIXOMOL treatment, however, in MDA-MB-231 cells the other compound, Br-HIMOLID showed higher activity. The results revealed that autophagy observed in studied cells was regulated by Beclin 1. Moreover, it was also concluded that HIXOMOL and Br-HIMOLID might induce autophagy in MCF7 and MDA-MB-231 cells by modulation of p53 and NF-κB transcription factors.

**Conclusions:** The newly synthesized derivatives of oleanolic acid induce autophagy in breast cancer cells in vitro and their effectiveness depends on cancer cell characteristics. HIXOMOL and Br-HIMOLID seem to be very promising compounds in the context of anticancer strategy and mechanism of their action requires further investigations.

The researchers were supported by grant from the National Science Centre, Grant No. 2011/01/N/NN4/03433

**Multiple ErbBs Inhibition by Lapatinib Blocks Tumor Growth in Orthotopic Model of Human Testicular Germ Cell Tumor**

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**Background:** The ErbB family of receptor tyrosine kinases plays important roles in cancer and many carcinomas present aberrant expression or activity of some of its members such as ErbB1 (EGFR) and ErbB2 (Neu). ErbBs have been involved in germ cell development, and in the case of ErbB1 it is overexpressed in testicular germ cell tumour (TGTS). These data together suggest a principal role of these proteins in the progression of the tumours, but still has to be demonstrated.

**Material and Method:** Using in vivo models of human non-seminomatous TGT orthotopically grown in nude mice, we analysed the ErbBs expression and the effect of the inhibitors cetuximab and gefitinib (ErbB1), lapatinib (ErbB1 and ErbB2). The newly synthetized derivatives of oleanolic acid induce autophagy in breast cancer cells in vitro and their effectiveness depends on cancer cell characteristics. HIXOMOL and Br-HIMOLID seem to be very promising compounds in the context of anticancer strategy and mechanism of their action requires further investigations.

**Results:** We found in vitro that HIXOMOL and Br-HIMOLID treatment in non-cytotoxic concentrations led to an induction of autophagic-programmed cell death. Observed activity of the compounds was dependent on cell characteristics. In MCF7 cells the autophagy process was expressed more intensively after HIXOMOL treatment, however, in MDA-MB-231 cells the other compound, Br-HIMOLID showed higher activity. The results revealed that autophagy observed in studied cells was regulated by Beclin 1. Moreover, it was also concluded that HIXOMOL and Br-HIMOLID might induce autophagy in MCF7 and MDA-MB-231 cells by modulation of p53 and NF-κB transcription factors.

**Conclusions:** The newly synthesized derivatives of oleanolic acid induce autophagy in breast cancer cells in vitro and their effectiveness depends on cancer cell characteristics. HIXOMOL and Br-HIMOLID seem to be very promising compounds in the context of anticancer strategy and mechanism of their action requires further investigations.

The researchers were supported by grant from the National Science Centre, Grant No. 2011/01/N/NN4/03433
**THc1 – a Rat-derived Parathyroid Continuous Cell Line Suitable to Study Parathyroid Cancer and Hyperplasia**

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**Introduction:** The parathyroid gland is one of the main organs involved in calcium (Ca²⁺) homeostasis, through the activation of the calcium-sensing receptor (CaSR), high extracellular calcium levels, leading to the inhibition of parathyroid hormone (PTH) secretion, PTH gene expression and parathyroid gland proliferation. Current knowledge of parathyroid gland physiology and pathology is mainly derived from studies on parathyroid tissue and primary cultures of bovine or human parathyroid cells because it is difficult to obtain continuous parathyroid cell lines that maintain functional characteristics similar to those of parathyrocytes. This study characterizes a continuous parathyroid cell line obtained from rat hyperplastic parathyroid tissue, able to express PTH and its receptor (CaSR) by high extracellular calcium levels, leading to the inhibition of the RafA/B effectors by immunoblotting analyses. We explored the Ha-ras1 silenced by methylation in human tumors and indicate that Nur77 is a p53-dependent tumor suppressor gene. Here we show that Nur77 is a transcriptional target of p53 that mediates some of its antioncogenic functions.

**Material and Methods:** Nur77 expression was studied in clinical samples by RT-qPCR. Silencing of the gene in human tumors was determined by methylation-specific PCR and sequencing of bisulfite-treated DNA. To study Nur77 regulation by p53 we used cell culture approaches. Nur77 promoter was cloned and luciferase experiments were performed in presence or absence of exogenous p53 as well as induction of endogenous p53. HTC116 wt and p53−/− (a gift from B Vogelstein) were used to show dependence of Nur77 induction (endogenous mRNA or reporter construct) on p53 presence. Apoptosis was induced in wildtype or Nur77-deficient mouse embryo fibroblasts (MEFs) with pharmaceutical treatments after sensitization with viral E1A oncoprotein. In vivo tumor studies were performed in Rb+/− mice crossed into a Nur77 null background (a gift from J Milbrandt). Tumor development was followed by MRI. Tumors were analyzed by RT-qPCR and H&H after necropsy.

**Results:** RT-qPCR analysis showed Nur77 is downregulated in several human cancers such as SCLC or gliomas. Moreover, low levels of Nur77 mRNA correlate with poor survival in gliomas. We have also demonstrated that epigenetic silencing by Nur77 gene methylation is a relevant mechanism explaining curtailed gene expression. We next focused on the relationship between Nur77 and p53. Our data show by different approaches that p53 stimulates Nur77 expression through a previously unidentified functional p53-response element. Furthermore, using cells from knockout mice we could also demonstrate that Nur77 is necessary for p53-dependent apoptotic responses upon treatment with common chemotherapeutic agents such as doxorubicin or cisplatin. We went further into showing the in vivo relevance of the functional interaction p53-Nur77 and demonstrate that inactivation of Nur77 in Rb+/− mice accelerates tumor development. Finally, pituitary tumors lacking Nur77 were less sensitive to treatment with bromocriptine, a dopamine agonist used in hypophysectomy.

**Conclusions:** Taken together our results show that Nur77 expression is silenced by methylation in human tumors and indicate that Nur77 is a p53-responsive gene that mediates, at least in part, p53-dependent apoptotic responses. This study was funded by Fundación de Investigación Medica Mutua Madrileña and Xunta de Galicia.

**DOXORUBICIN CTX TOXICITY IN ENDOMETRIAL CANCER – IMPACT OF STEM CELLS**

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**Background:** Endometrial cancer is one of the most common female malignancies in the world and the seventh leading cause of death of women in the European Union. The existence of cell populations with characteristics of stem cells (CSC’s) that are resistant to conventional therapies such as chemotherapy, represent a drawback to treat this kind of cancer. This project aims to identify CSC and to verify their implication in response to chemotherapy.

**Material and Methods:** The human endometrial cancer cell line ECC-1, was propagated at 37°C and 5% CO₂ using RPMI-1640 medium supplemented with 5% fetal bovine serum. To determine the sphere forming assay cells were seeded in non-adherent culture conditions in DMEM/F12 supplemented daily with basic fibroblast growth factor and epidermal growth factor. ECC-1-G cells were obtained putting CSC’s in adherent conditions. CSC’s CD24, CD44, CD45

**Results:** The EVOO diet, rich in the monounsaturated fatty acid oleic acid and diminished the mono-ubiquitlated PCNA levels, which is related to DNA damage. Tumors from rats fed the EVOO diet developed a more benign phenotype, whereas those from rats fed the HCO diet were biologically more aggressive. Conclusion: High EVOO and corn oil diets exert their differential modulatory effects on breast cancer through a different combination of Ras signalling pathways, a different proliferation–apoptosis balance and probably distinct levels of DNA damage.

**Nur77 as a Tumor Suppressor That Mediates P53 Antioncogenic Activities**

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**Background:** Recent work has shown that orphan nuclear receptor Nur77 is a key molecule in regulation of apoptosis. Desregulation of Nur77 has been described in certain cancer types and although there is contradictory data in the literature, evidence from a double knockout mouse model has supported the hypothesis that Nur77 functions as a tumor suppressor gene.

**Materials and Methods:** Cell cultures, subcloning: PTHc1 epithelial cell line was generated as described. Cell growth analysis: Growth curves were performed by counting the number of cells for 15 days. Gene expression: Gene expression was performed by qualitative RT-PCR. Immunocytochemistry: For immunofluorescence cells were stained for PTH, actin, and nuclei.

**Results and Discussion:** Cell clones maintained a polygonal shape, even after 12 months in culture, confirming the maintenance of morphologic characteristics. The PTHc1 clone presented a population doubling time of 23h. The expression of PTH, PTHR, PTHLRR, ERα, GR, GH-R, HRT2, LRP-5, VDR, CaSR, MKN-1, SFRP4, PHEX, PRAD1, GALNT3, IL6, NPT2a and 1α-HYDROXYLASE was found in the PTHc1 cell line, confirming the presence of characteristic parathyroid genes. A laser scanning confocal microscopy analysis in PTHc1 cells highlighted the presence of actin filaments well-formed of characteristic parathyroid genes. A laser scanning confocal microscopy was found in the PTHc1 cell line, confirming the presence of actin filaments well-formed of characteristic parathyroid genes. A laser scanning confocal microscopy analysis in PTHc1 cells highlighted the presence of actin filaments well-formed of characteristic parathyroid genes.

**Conclusion:** This study has investigated mechanisms of these differential modulatory actions on breast cancer, unlike high corn oil (HCO) diet that stimulate. We have investigated mechanisms of these differential modulatory actions on breast cancer.
and CD133 expression was assessed by flow cytometry in ECC-1 cell line and in cell with properties of stem cells. In order to establish dose-response curves cells (ECC-1 and ECC-1-G) were incubated with several concentrations of doxorubicin, ranging from 0.05 nM to 37 nM. After 24, 48 or 72 hours of incubation the cytotoxicity was evaluated by MTT or Alamar Blue assays.

### Results and Discussion

**Identification of a Tumour Suppressor MiRNA That Correlates to Therapy Response in Breast Cancer**


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2. Clinical Research Facility and School of Mathematics Statistics and Applied Mathematics, National University of Galway, Ireland, Co. Galway, Ireland

**Introduction:** MicroRNAs are small non-coding RNA molecules that control gene expression post-transcriptionally. MicroRNA expression has been found to be altered in many diseases including breast cancer. This has raised their potential as a biomarker for the disease. An ideal biomarker has the capacity to improve diagnostic, prognostic classification and prediction of the disease. The aim of this study was to quantify the expression of a particular miRNA of interest (miR-A*) and investigate a correlation with Retinoic acid receptor beta (RARβ) gene expression and clinicopathological details.

**Materials and Methods:** Following informed patient consent and ethical approval, tissues were harvested from patients undergoing surgery (breast cancer, n=68, normal n=30 and benign n=14). The tissues were homogenised in Trizol and total (small and large) RNA was extracted. MicroRNA was reverse transcribed and the level of miR-A in all tissue samples quantified using RT-qPCR relative to the expression of the endogenous control hsa

| Key
| Value |
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Data revealed a significant decrease in miR-A in breast cancer tissue (n=68, Mean (SEM) 2.29 (0.09) Log10 Relative Quantity (RQ)) compared to healthy controls (n=30, 2.95 (0.16) Log10 RQ, p < 0.001), with no significant difference observed between benign (n=14, 3.45 (0.3) Log10 RQ) and normal tissue. Investigation of patient clinicopathological details revealed a significant relationship between miR-A levels and tumour stage and grade.

**Conclusion:** The current study demonstrates a correlation between levels of miR-A and breast cancer stage and grade. The relationship observed between miR-A and commonly used prognostic indicators (e.g., tumour size and lymph node status) suggests that miR-A may be a potential biomarker for breast cancer prognosis.

**Predictive Value of CD44, CD24, CD133 and P-glycoprotein for Response to Treatment With a Pan ErbB Blocker and Gemicitabine in Pancreatic Tumour Cells**

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2. St George’s University of London, Cellular and Molecular Medicine, London, United Kingdom

**Introduction:** Pancreatic cancer is one of the most aggressive types of human cancer with drug-resistance being a major cause of treatment failure. Cancer Stem Cells (CSCs) have been indentified in several types of human malignancies and are believed to play an important role in tumour initiation, progression and resistance to therapy. The aim of this study was to investigate the expression levels and predictive value of putative CSCs markers (CD44, CD24 and CD133) and drug-resistance antigen p-glycoprotein (Pgp) in a panel of pancreatic cancer cells (BxPc3, AspC-1, Capan-1, PAN-1, FAD, MiaPaca-2, PT-45) for response to treatment with an irreversible HER family blocker and cytotoxic drugs. In addition, we investigated the changes in the expression pattern of the above markers as well as HER family members in BxPc3 cells that acquired resistance to afatinib or gemcitabine.

**Materials and Methods:** The expression of cell surface antigens was determined by flow cytometry. Drug-resistant BxPc3 cells were developed following treatment with increasing doses of gemcitabine or afatinib. Growth inhibition studies were performed using the sulforhodamine B (SRB) colorimetric assay.

**Results and Discussion:** All human pancreatic tumour cell lines were CD133 negative and, with the exception of BxPc3 and PANC1, were either negative or expressed very low levels of CD24. Similarly, the levels of Pgp were very low for the majority of the cell lines examined. In contrast, the great majority of pancreatic cancer cell lines expressed high levels of CD44. However, there was no clear association between CD44 or CD24 expression and response to treatment with afatinib, gemcitabine, 5-FU or doxorubicine. Of the two newly established drug resistant BxPc3 cell lines, afatinib-resistant (BxPc3AFR) and gemcitabine-resistant (BxPc3GEMR) cell lines exhibited >80-fold increase in IC50 for afatinib and gemcitabine respectively. Interestingly, BxPc3AFR also acquired resistance to gemcitabine and this was accompanied by an increase in the CD44 expression. BxPc3GEMR cells also acquired resistance to afatinib and this was accompanied by a decrease in the expression levels of CD24.

**Conclusion:** Our results suggest there is no association between the expression levels of CD44, CD24 and CD133 in pancreatic cancer cell lines and response to treatment with the pan-HER inhibitor afatinib and cytotoxic drugs. However, acquired resistance of pancreatic tumour cells to afatinib is accompanied by changes in the expression levels of CD44 and warrants further investigation.

**Three-Dimensional Co-culture of Mesenchymal Stem Cells With Colorectal Cancer Cells in Vitro Has A Distinct Effect on Cytokine Secretion**

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**Introduction:** Mesenchymal Stem Cells (MSCs) are multipotent stromal cells known to home to colorectal cancers (CRC) and integrate into the tumour architecture. However, their role following engraftment has yet to be elucidated. They are thought to exert their effects through secretion of a range of cytokines that influence key properties of the tumour. This study aimed to investigate interactions between MSCs and human CRC cells in three-dimensional culture, and identify the factors involved.

**Methods:** Cultures were established from CRC cell lines (HCT-116 and HT 29) alone, or in combination with MSCs or normal fibroblasts (WI 38), on 3-D scaffolds. MSCs were more closely associated with the in vitro experiments. Cells were seeded at two different ratios of CRC cells to stromal cells (3:1 and 30:1). Conditioned media containing all secreted factors was harvested at day 1, 3 and 7, and the range of cytokines secreted by individual and co-cultured populations detected using ChemiArray. The level of specific factors of interest was then quantified using ELISA.

**Results:** ChemiArray analysis revealed that all cell populations secreted a range of cytokines, and upon co-culture, Plasminogen Activator Inhibitor-1 (PAI-1) and Macrophage Migratory Inhibitory Factor (MIF) were selected for further analysis. High levels of both of these factors have previously been shown to correlate with poor outcome in CRC. HCT-116, the more invasive CRC cell line, secreted higher levels of PAI-1 (range 0–4 ng/mL) than HT-29 cells (0–1 ng/mL). The highest levels of PAI-1 were secreted by stromal cell populations (MSC: 2–18 ng/mL, WI-38: 1–18 ng/mL). Upon co-culture of MSCs or WI-38s with HCT-116 cells, there was a significant decrease (20–88%) in PAI-1 secretion detected across all timepoints. This effect was greater in the presence of higher numbers of cancer cells. A similar effect was observed with HT-29 cells, with PAI-1 secretion decreased upon co-culture with MSCs (mean decrease 25%). In the case of MIF, the highest levels were secreted by the stromal cell line HCT-116 (range 14–52 ng/mL), whereas PAI-1 secretion increased 80-fold (range 1–4 ng/mL). For both CRC cell lines, similar to PAI-1, co-culture with MSCs resulted in a net decrease in MIF secretion (21–90% decrease). Interestingly, co-culture with normal fibroblasts (WI-38) had no significant effect on MIF production, with a large increase in secretion of PAI-1 and MIF observed upon co-culture with CRC cell lines, suggesting an MSC specific phenomenon.

**Conclusion:** As a component of colorectal cancers, MSCs may have a significant impact on tumour biology. This data highlights distinct effects of MSC co-culture on PAI-1 and MIF levels in the CRC cell microenvironment in vitro. Considering the association of both cytokines with CRC progression, further elucidation of the effects of MSCs in this microenvironment is crucial.
Inhibition of Phosphatidylcholine-specific Phospholipase C Enhancing 5-FU Activity in Colorectal Carcinoma-derived Cell

A Nude-mice Xenotransplant Model for Studying the Early S.Cecchetti2, E. Iorio2, S. Miotti1, F. Podó2, S. Canevari1.

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S100 MCT inhibitors (as new therapeutic approaches in CRC. For that purpose, we used two CRC-inhibitors and encapsulation into zeolites and exploring these methodologies systems (DDSs).

Different drug molecules into them so as to obtain subsequent drug delivery many potentially medicinal applications of zeolites is the encapsulation of biological properties and stability in biological environments. One of the lactate transporters, namely monocarboxylate transporters (MCTs). Zeolites of lactate that must be released to the extracellular microenvironment by (5-FU) is one of the most efficient chemotherapeutic agents in the treatment

Conclusion: These results suggest a role for PC-PLC in stem cells maintenance and, if confirmed, may open the way to new molecular therapeutic strategies aimed at selective interference of TIC proliferation and survival.

Enhancing 5-FU Activity in Colorectal Carcinoma-derived Cell Lines - Combination With Monocarboxytyl Transporter Inhibitors and Encapsulation into Zeolites R. Amorim1, N. Vilaça2, A.M. Fonseca2, I.C. Neves2, A. Preto2, F. Baltazar2. 1Life and Health Sciences Research Institute (ICVS), ICVS/3B’s – PT Government Associate Laboratory, Braga/Guimarães, Portugal, 2Center of Chemistry, Chemistry Department University of Minho, Braga, Portugal, 3Centre of Molecular and Environmental Biology, Department of Biology University of Minho, Braga, Portugal

Colorctal cancer (CRC) is the second most common type of cancer worldwide, being mainly a disease of industrialized countries. 5-Fluorouracil (5-FU) is one of the most efficient chemotherapeutic agents in the treatment of CRC although resistance to 5-FU treatment has been reported. CRC cells rely on glycolysis to obtain energy thus producing large amounts of lactate that must be released to the extracellular microenvironment by lactate transporters, namely monocarboxylate transporters (MCTs). Zeolites are crystalline aluminosilicates solids with very regular microporous structures and they have been recently considered for medical use due to their biological properties and stability in biological environments. One of the many potential levels of drug resistance is the encapsulation of different drug molecules into them so as to obtain subsequent drug delivery systems (DDSs).

This work aimed at enhancing the activity of 5-FU by the combined use of MCT inhibitors and encapsulation into zeolites and exploring these methodologies as new therapeutic approaches in CRC. For that purpose, we used two CRC-derived cell lines, HCT-15 and RKO, and assessed the effects of combining MCT inhibitors with 5-FU in these CRC cell lines. Our results showed that the combination of MCT inhibitors with 5-FU resulted in an increase of 5-FU cytotoxic effect in both cell lines. We were also able to observe that, while no overall toxic effect was observed upon incubation of zeolites alone in both cell lines, the incorporation of 5-FU into the zeolites resulted, in general, in an increase of the cytotoxicity as well as a higher inhibitory effect on the biomass of both cell lines, as compared to 5-FU alone. Our findings provide important evidence for the exploitation of MCT inhibitors in the pharmacological therapy of CRC since we showed a benefit of combining these drugs with the conventional anticancer drug 5-FU. This preliminary study provides evidence that zeolites constitute good systems for drug delivery in in vitro models of CRC, which can be useful in future cancer therapies.

Phenotypic and Functional Characterization of an Invasive Subpopulation of MDA-MB-231 Breast Cancer Cell Line G. Angelini1, A. Amaro1, A. Esposito1, M. Maffei1, W. Giaretti1, S. Astigiano2, O. Barbieri1, M. Viale1, A. Zunino1, U. Pfeffer1, 1IRCCS AOI San Martino – IST, Integrated Molecular Pathology, Genova, Italy, 2IRCCS AOU San Martino – IST, Embryogenesis and Tumorigenesis in Animal Models, Genova, Italy.

Introduction: The acquisition of an invasive phenotype is a prerequisite for metastatization, yet it is not clear whether or to which extent the invasive phenotype is linked to other features that are specific for metastatic cells. Materials and Methods: We selected an invasive subpopulation from the triple negative breast cancer cell line MDA-MB-231 through repeated cycles of preparative assays of invasion, through Matrigel covered membranes. The selected ‘invasive’ population of MDA-MB-231 cell was also maintained in culture for six months to investigate if the invasive phenotype is transient or stable. Phenotypic and functional characterization of the three cell lines: the parental, the ‘invasive’ sub-population and the ‘long term’, was done. Deep genotypic analysis was performed by the gene expression profiling, by the analysis of copy number variations and by karyotypic analysis. The grade of ploidy was assessed by DNA index. Chemo-sensitivity assays, and analysis of the proliferation and apoptosis rate were also performed, while characterization of the metastatic potential of the invasive cells in vivo is presently ongoing.

Results and Discussion: The invasion assay of the sub-population of MDA-MB-231 cells confirmed the highly invasive phenotype of the selected line. Prolonged cultivation of these cells did not abolish the invasive phenotype. Genetic analyses revealed many genetic alterations in the invasive cell line including increased ploidy, confirmed by DNA index quantification and karyotype analysis. The invasive cells proliferate and undergo apoptosis similar to the parental cell line. Commitment to apoptosis is increased since invasive cells respond more strongly to drug induced apoptosis. The invasive phenotype is not related to stem cell features nor to epithelial mesenchymal transition. Invasive cells show an altered pattern of chemo-sensitivity with lower IC50 values for drugs affecting the mitotic apparatus. Orthotopic xenografts of the different cell populations in nude mice has been done and the experiment is presently underway.

Conclusion: We selected a stable, highly invasive subpopulation from the breast cancer cell line MDA-MB-231. The final goal is to investigate into the prognostic potential of invasion related gene expression profiles in order to develop an invasivity signature, able to predict relapse for human breast cancers.

A Nude-mice Xenotransplant Model for Studying the Early Stages of Tumor Angiogenesis of Human Osteosarcomas J.A. Lopez-Guerrero1, F. Giner2, I. Machado3, Z. Garcia-Cassado1, A. Peydo-Olaya1, A. Llombart-Bosch2. 1Fundacion Instituto Valenciano de Oncologia, Laboratory of Molecular Biology, Valencia, Spain, 2Universitat de Valencia Estudis General, Department of Pathology, Valencia, Spain, 3Fundacion Instituto Valenciano de Oncologia, Department of Pathology, Valencia, Spain

Background: Between 25-50% of osteosarcoma (Os) patients develop metastatic disease after current treatment, which remains the major cause of death. Currently, there is considerable interest in the therapeutic potential of targeting angiogenesis. Whereas angiogenic inhibitors have been used increasingly in a number of clinical trials for the treatment of advanced cancers, their potential role as an adjuvant to chemotherapy in osteosarcoma, a hypervascular tumor, is still unclear. The aim of this study was to characterize the angiogenic potential of MDA-MB-231 cells, their sensitivity to antiangiogenic drugs, and to test the angiogenic potential of these cells in vivo.

Material and Methods: Three xenotransplanted human Os were evaluated: Nu399, Nu498 and Nu499. Tumor pieces of 0.3-0.4 cm in size were implanted into the backs of nude mice (albino Balb-c nude mice) which were sacrificed at 48, 72 hours p.s. 14, 21 and 28 days from implantation. The tumor was cut and divided into three pieces for electron microscopy (EM), histology and molecular biology. The expression of angiogenic factor was evaluated by immunohistochemistry (IHC) and quantitative RT-PCR by means of using
Results: We observed that after 24–48 hours from implantation all tumors expressed high levels of several angiogenic factors, especially VEGF and their receptors, FGFR, CXCL9 and GRO. At this point the stroma surrounding the tumor initiates an angiogenic induction producing capillary congestion. Co-option between endothelial cells and tumor cells together with pseudo-vessels containing only tumor cells (vascular mimicry) was also observed. Western analysis showed that the expression profiles at 48h and 1 week had the same behavior and in turn these correlated with the angiogenesis induction and the angiogenic remodeling within the tumor respectively. Human VEGF was not reported in the blood of any of the analyzed mice. However, the serum levels of mouse VEGF were higher after the first week from tumor implantation.

Conclusions: The model herein presented offers an excellent way in which to study the initiation process of tumor angiogenesis and provides new ways to assess the activity of potential inhibitory agents associated with tumor growth.

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**[416] Cancer-testis Antigen MAGE-C2/CT10 Promotes Proliferation and Resistance to Apoptosis in Multiple Myeloma**

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Background: TRIM59 belongs to the TRIM family of proteins. They share structural similarities and are involved in several biological functions, such as gene expression or protein degradation. Little is known about TRIM59 function, although recent studies have suggested that TRIM59 could regulate cell proliferation. Our previous results have shown that TRIM59 expression is increased in tumor samples from mouse models of cancer. Here we address the hypothesis that TRIM59 could act as an oncogene in humans.

Material and Methods: RT-qPCR was used to determine TRIM59 expression in human tumor samples. To study proliferation, cell cycle profiles and checkpoint activation upon Traf59 overexpression, Hek293, Saos2 and U2OS cells were transiently transfected with a TRIM59-YFP fusion construct and YFP-positive cells were followed over time. Immunofluorescence staining of endogenous TRIM59 was achieved by using an antibody against TRIM59 and a secondary antibody conjugated to Alexa Fluor 594.

Results: To explore the oncogenic function of TRIM59, we first studied its expression in different human tumor types. In agreement with the results in animal models, we found higher levels of TRIM59 mRNA in non-small cell lung carcinomas, colon tumors and gliomas. Moreover, the expression level of patients with glioma is low when TRIM59 levels are high. To study the effects of differential TRIM59 expression in human cell lines we did transient overexpression studies, as well as gene silencing in cell cultures. In different cell lines, ectopic expression of TRIM59 caused alteration in cell cycle profile with S/G2-M accumulation, as well as activation of markers of the DNA damage response, a finding consistent with oncogenic stress induced upon activation of well-characterized oncogenes. Finally, TRIM59 silencing in the A549 cell line caused an impaired response to cisplatin treatment, as well as defects in the epithelial-to-mesenchymal transition induced by TGF-β.

Conclusions: Our results suggest that TRIM59 acts as an oncogene in human tumors and that its function could be related to DNA damage response and EMT induction.

**[418] Alternative Splicing Variants BRCA1α14−15 and α17−19 Differentially Impair the DNA Double Strand Break Response of MCF-7 Cells**

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Mutations in the BRCA1 gene are responsible for the majority of hereditary breast cancer (BC) cases in the Czech Republic. During the screening of high-risk BC individuals, various alternative splicing variants of BRCA1 with unknown clinical significance have been repeatedly ascertained. To evaluate their biological importance, we developed cell line-based in vitro system.

Material and Methods: The in-frame BRCA1 alternative splicing variants Δα14−15 and Δα17−19 were expressed from pDNA3.1 vector in MCF-7 cell line with/without coincidentally downregulated expression of wt BRCA1 using a psUPER-shRNA vector. Functionality of expression systems was confirmed on both the mRNA (qPCR) and protein levels (western blotting). A DNA double-strand break (DSBB) repair assay was performed. In all clones with modified expression of BRCA1, the activity of NHEJ was significantly lower than in controls. The activity of homologous recombination (HR) and non-homologous end joining (NHEJ) assay, respectively. A Clonogenic assay was used for the evaluation of radiation sensitivity of MCF-7 clones with modified BRCA1 expression.

Results and Discussion: Both DSBB repair capacity and IRIF kinetics were significantly decreased after IR in clones expressing BRCA1α14−15 and BRCA1α17−19. Clones with upregulated expression of BRCA1α14−15 and Δα17−19 showed no difference in sensitivity to mitomycin C in comparison with controls with downregulated or stable wtBRCA1 and controls. In all clones with modified expression of BRCA1, the activity of NHEJ was significantly lower than in controls. The proliferation of cells examined in relation to IR-induced DNA damage showed that clones expressing BRCA1α17−19 but not BRCA1α14−15 exert increased radiation resistance.

Conclusions: Our current results indicate that in some aspects the overexpression of BRCA1α14−15 (lacking a portion of Ser-containing domain) and BRCA1α17−19 (lacking the first BRCT domain) has a similar impact...
on DDSB response as the depletion of wBRCA1. Contrary to wBRCA1- downregulation affecting both HR and NHEJ repair, the expression of BRCA1.14–15 and 1.17–19 variants showed predominant HR repayment. We conclude that both variants negatively affect genomic stability but solely BRCA1.17–19 influences the clonogenic potential in 3D cells. Acknowledgement: grants GACR P301/12/1850; IGA NT12280; GAUK 428711.

[41] USER-based Approach for Identification of BRCA1 Alternative Splicing Variants
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Background: BRCA1 is a multifunctional protein with a key role in regulation of DNA double strand break repair. Alterations in the BRCA1 gene are responsible for breast ovarian cancer susceptibility. Except for the aberrant splicing emerging from the BRCA1 gene alterations, endogenously expressed alternative splicing variants (ASVs) have been described in various cell lines and tissues. Some of these represent in-frame ASVs that can be translated into the protein isoforms lacking crucial functional domains of BRCA1 coded by spliced exons. With a growing need to understand the significance and function of BRCA1 and its isoforms, a robust tool for identification of BRCA1 ASVs is required since their catalogue has not been established yet. Here we present an approach for the identification of BRCA1 ASVs.

Material and Method: cDNA from MCF-7 cell line was amplified with uracil-containing specific primers and cloned into the pNEB206A vector using uracil-specific excision reagent (USER Friendly Cloning Kit; NEB). Created constructs were used for transformation of TOP10 E.coli competent cells. Colonies selected using the IPTG/Gal screening were cultivated in 96-well plate for 4 h and used for direct PCR screening with BRCA1-specific primers and subsequent sequencing.

Results and Discussion: We identified 28 individual ASVs of BRCA1 mRNA in MCF-7 cells. Ten in-frame ASVs lacked exons coding for functionality important BRCA1 domains including the DNA-interaction domain (Δ10b), nuclear localization signal (Δ9) or RING-finger domain (Δ10b). These variants may have a critical impact on resulting BRCA1 function. Eleven exons ASVs exhibited frame-shift leading to premature termination of translation and 7 ASVs with large deletions (Δ2–14) that lacked exon 2 containing the initiation codon. Therefore, the translation of these ASVs was considered improbable. Since BRCA1 mRNA occurs in (individual cell) in a number of ASVs which differ also in short (3–21 bp) in-frame deletions, the identification of such complex splicing pattern by gel electrophoresis and subsequent sequencing is not possible. The use of next-gen transcriptome analysis is also not suitable due to the presence of the large exon 10a (3426bp) and ASVs with complex exon composition (e.g. Δ4+Δ8+9+14b).

Conclusion: The USER-based cloning of individual mRNA variants represents robust and reliable system for identification of complex BRCA1 mRNA splicing.

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[42a] A Platform of Porous Biomaterials as 3D Culture Systems for Cancer Biology
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Background: 3D scaffolds offer a new platform to study the mechanisms behind stem cell driven tissue morphogenesis and may play a role in cancer biology research to create organotypic 3D models to study cancer initiation and development, as well as the potential involvement of stem cells in these processes.

[42b] Ethanol Directly Modulates Reactive Oxygen Species Generation In Oral Squamous Cell Carcinoma Cell Lines
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Background: Oral cancer is the sixth most common cancer in the world and has one of the highest death rates, due to it being largely asymptomatic until the latter stages of the disease. It is a major worldwide public health problem with the incidence and mortality rising in several regions of the world, including Europe, South Central Asia and Australia. Smoking poses the highest risk for oral cancer followed closely by the consumption of alcohol. This study aims to further understand the role of ethanol in the carcinogenic transformation of oral tissue with the specific interest in reactive oxygen species (ROS) production and the effect on cell cycle progression in oral cavity squamous cell carcinoma cell lines.

Materials and Methods: Two oral cancer cell lines were utilised as models for this study. Ca9-22 (gingival) cell line and TR146 (buccal mucosa) cell line. Cells were cultured in the presence or absence of a range of ethanol concentrations over 6, 24 and 48h time points. Cell viability was determined using Alamar Blue. Alcohol dehydrogenase assays were performed to monitor the activity of the enzyme within the cell and to ensure the absence of the enzyme in the cell culture medium. Extracellular production of hydrogen peroxide (H2O2) was monitored using Ampex Red and intracellular production of H2O2 was monitored using 2′,7’-dichlorofluoresceindiacetate (DCFH-DA) and analysed by both flow cytometry and confocal microscopy. Perturbations in the cell cycle were analysed with flow cytometry. Expression levels of proteins that control the cell cycle were determined by western blotting. The levels of expression of specific genes underlying the control of the hypoxic response in the cell were determined by quantitative real time polymerase chain reaction.

Results: Ethanol concentrations from 0.1–3% showed no detrimental effect on cell survival over the given time course (n=8). The presence of ethanol was found to reduce the release of H2O2 in a dose dependent manner (p<0.05–0.001 in relation to control, n=3) however when normalized for cell numbers over the duration of the study a biphasic response was observed. Cell cycle analysis revealed a biphasic trend at the lower concentrations of ethanol with no significant reduction in the progression index at the time point examined. No significant induction of apoptosis or polyplody events were observed.

Conclusion: These findings suggest that the significant reduction in the production of cellular H2O2 is as result of ethanol and not its first metabolite acetaldehyde, which is a known carcinogen. Lack of induction of apoptosis may allow for both low levels of ROS and ethanol to cause progressive damage to DNA. This alteration in oxidative stress could induce a potential switch from apoptotic to necrotic cell death which may lead to eventual carcinogenic transformation.

[42c] Investigation of the Apoptotic Pathways Activated by Equol and Tamarixan in MCF-7 Breast Cancer Cells
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Background: Soy phytoestrogens, such as daidzein and its metabolite equol, have been proposed to be responsible for the low breast cancer rate in Asian women. However, since these compounds possess both estrogenic and anti-estrogenic properties, it remains unknown whether they provide protection against tumor development and progression. Since the majority of estrogen receptor positive (ER+) breast cancer patients are treated with the selective estrogen receptor modulator tamoxifen, the objective of this study is to determine whether equol enhances or inhibits tamoxifen’s anti-tumor effect and to identify the molecular mechanisms involved. For this purpose, we examined the individual and combined effects of equol and tamarixan on the ER+ breast cancer cell line MCF-7.

Materials and Methods: In order to examine the effects of equol and tamarixan on the MCF-7 cells, we used viability assays (MTT assay), cell cycle analysis, annexin-V/PI staining, cell death ELISA, and western blot analysis. The effects of equol and tamarixan on the expression of pro-apoptotic and anti-apoptotic markers were determined using western blot analysis. The analysis of gene expression was performed using qRT-PCR. The results were compared to the control group and the statistical significance was determined using the Student’s t-test. The data obtained were subjected to analysis by the Mann-Whitney U test. The results were considered significant if the p-value was less than 0.05.
**Acquisition of Resistance to Anti-Her2 Therapies Promotes a Role of the Ubiquinone Site of Complex II in Apoptosis Induction**

**Results:** We found that equol at concentrations higher than 10 μM significantly reduced the viability of MCF-7 cells. Furthermore, the combination of equol (100 μM) and 4-hydroxytamoxifen (10 μM, 4OHT) reduced MCF-7 cell viability and induced apoptosis more effectively than each compound alone. Subsequent treatment of MCF-7 cells with the pan-caspase inhibitor Z-VAD-FMK demonstrated that equol and 4-OHT induce apoptosis predominantly via caspase-dependent mechanisms. In order to further investigate the apoptotic pathways employed by equol, 4-OHT and their combination, we evaluated the expression of key pro-apoptotic and anti-apoptotic proteins such as PARP, α-fodrin, AIF, bcl-2, bax, p21 and p53 using Western blot analysis. The combination of 4-OHT and equol induced PARP and α-fodrin cleavage which was inhibited by Z-VAD-FMK indicating that their combination induces caspase-dependent apoptosis. This was accompanied by reduced bcl-2: bax ratio confirming a pro-apoptotic effect. Equol and 4-OHT, individually and combined, induced caspase-9 and caspase-7 cleavage as well as cytochrome-c release to the cytosol, indicating activation of the intrinsic apoptotic pathway in equol and tamoxifen induced apoptosis. Finally, upon determination of the effect of equol and tamoxifen on estrogen receptor expression in MCF-7 cells, it was found that equol inhibited the 4-OHT induced increase in the ERα:ERβ ratio, providing an explanation for the beneficial combined effects of equol and tamoxifen.

**Conclusions:** In conclusion, the effects of equol in potentiating tamoxifen’s anti-tumor action may be explained by the induction of the caspase-mediated apoptotic pathway and the down regulation of ERα. These results can find applications in combination cancer chemoprevention studies against breast cancer.

**Distinct MiRNA Expression Patterns in Among Breast Tumors in Different Microenvironment Crosstalk Capability**

**Introduction:** Mechanisms underlying tumor progression after chemotherapy are not well understood. Therapeutic treatments can favor the clonal selection of cells with unique properties and different fitness for a given microenvironment. Indeed, tumor cells can induce changes in the structure and composition of the microenvironment to support their growth and spread. **Materials and Methods:** In order to investigate if clonal selection induced by target therapies like Trastuzumab and Lapatinib favors the outgrowth of cells with different capability of microenvironment crosstalk, we developed different cell lines resistant to these drugs from the parental SKBR3, BT474 and MDAMB-453Her2+ cell lines. We determined their molecular profile and identified the drugs and combinations that favor the outgrowth of resistant cell lines. These results can find applications in combination cancer chemoprevention studies against breast cancer.

**Role of the Ubiquinone Site of Complex II in Apoptosis Induction and Malignant Transformation**

**Introduction:** Mitochondrial complex II (CII) comprises four subunits classified as tumour suppressors. We identified the proximal UbQ-binding site (Qβ) of CII as a target for the mitocan α-TOS and its more effective mitochondrialy derivated derivative MitoVES. To better characterise this interaction, we prepared cell lines mutated in several residues lining the Qβ of which RT2 and I56 are mutated in paraganglioma. These cell lines were made malignant by stable transfection with H-Ras. Malignant cells with specific SDHC mutations defined the association between expression status of Let-7a and miR-335 and breast cancer. This is the first study evaluating miRNA expression patterns in breast tumors of patients carried and non-carryed germ-line BRCA mutations.

**Results:** SDHC-deficient B9 cells were stably transfected with human SDHC carrying point mutations in residues S68, I56, RT2 and I69, and then made malignant by stable transfection with H-RAS. Native electrophoresis was used to assess the impact of the mutations on the assembly of CII. Two distinct enzymatic activities of CII (succinate dehydrogenase, SDH and succinate quinone reductase, SQR) were assessed spectrophotometrically. Apoptotic response to MitoVES was evaluated by flow cytometry. Long term cell culture experiments to determine the transforming potential of the mutations are ongoing.

**Results and Discussion:** When compared to parental B9 cells, CII assembly was restored in SDHC transfected cells, though the assembly was not complete in several mutants. Despite the presence of assembled CII, the SQR activity was compromised in all mutated cell lines, except for cells with mutations of the I56 residue. While the SQR activity was completely lost in the S68L, I56E and RT2C mutants, their SDH activity was preserved, albeit mostly to a lower degree compared to wt SDHC cells. In general, both SDH and SQR activities were lower in malignant cells. The apoptotic response to MitoVES treatment was lower in H-RAS transformed cells with mutations in SDHC (except for I56E and I56K) and in all non-malignant cells.

**Conclusion:** The I56F and I56V mutations caused relative resistance to apoptosis induced by MitoVES, strongly implicating the Qβ site in cell death triggered by the agent. Further, resistance of non-malignant cell lines confirmed the selectivity of the drug for malignant cells. Evaluation of the CII activities suggested that defects in CII become more prominent in the H-Ras transformed cells, which also have a lower level of assembled respiratory supercomplexes, which might be associated with less efficient mitochondrial respiration.

**Distinct MiRNA Expression Patterns in Among Breast Tumors in Different Microenvironment Crosstalk Capability**

**Results:** We found that equol at concentrations higher than 10 μM significantly reduced the viability of MCF-7 cells. Furthermore, the combination of equol (100 μM) and 4-hydroxytamoxifen (10 μM, 4OHT) reduced MCF-7 cell viability and induced apoptosis more effectively than each compound alone. Subsequent treatment of MCF-7 cells with the pan-caspase inhibitor Z-VAD-FMK demonstrated that equol and 4-OHT induce apoptosis predominantly via caspase-dependent mechanisms. In order to further investigate the apoptotic pathways employed by equol, 4-OHT and their combination, we evaluated the expression of key pro-apoptotic and anti-apoptotic proteins such as PARP, α-fodrin, AIF, bcl-2, bax, p21 and p53 using Western blot analysis. The combination of 4-OHT and equol induced PARP and α-fodrin cleavage which was inhibited by Z-VAD-FMK indicating that their combination induces caspase-dependent apoptosis. This was accompanied by reduced bcl-2: bax ratio confirming a pro-apoptotic effect. Equol and 4-OHT, individually and combined, induced caspase-9 and caspase-7 cleavage as well as cytochrome-c release to the cytosol, indicating activation of the intrinsic apoptotic pathway in equol and tamoxifen induced apoptosis. Finally, upon determination of the effect of equol and tamoxifen on estrogen receptor expression in MCF-7 cells, it was found that equol inhibited the 4-OHT induced increase in the ERα:ERβ ratio, providing an explanation for the beneficial combined effects of equol and tamoxifen.

**Conclusions:** In conclusion, the effects of equol in potentiating tamoxifen’s anti-tumor action may be explained by the induction of the caspase-mediated apoptotic pathway and the down regulation of ERα. These results can find applications in combination cancer chemoprevention studies against breast cancer.
The Major Vault Protein (MVP) Mediates Starvation Resistance of Human Glioblastoma Cells Via Deregulation of the PI3K/AKT Pathway

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Background: Vaults are highly conserved ribonucleoprotein particles ubiquitously expressed in eukaryotic organisms. They predominantly consist of the 110 kDa major vault protein (MVP) and have been implicated in the regulation of multiple cellular processes including transport mechanisms, chemoresistance, and intracellular signalling pathways. While in normal brain the expression is low, MVP levels are consistently upregulated in glioblastoma multiforme (GBM). Aim of this study was to investigate whether MVP/vaults have an impact on GBM cell growth and survival, including chemotherapeutic responsiveness, and to clarify underlying molecular mechanisms.

Material and Methods: The MVP protein was stably overexpressed in MVD-G and MVD-CS cells by transfection of cDNA for wild-type or a truncated protein. The truncated protein was targeted to the Golgi apparatus and secreted extracellularly to signal with a differential subcellular localization. Western blot analysis confirmed the expression of MVP and proMVP proteins.

Results and Discussion: Extracellular MVP expression in H7 glioma cells did not substantially alter sensitivity against diverse chemotherapeutic drugs. However, responsiveness to growth factor stimulation (EGF, serum) was increased parallelled by a significant upregulation of MAPK- and PI3K-pathway indicated by phosphorylation of ERK, AKT and S6. Moreover, MVP-transgenic cells were progressively resistant to apoptotic cell death induced by serum-starvation, an effect reversible by shRNA-mediated MVP-repression. PI3K downstream signalling, namely AKT and S6 phosphorylation, was hyperactivated in MVP-positive as compared to control transfected cells. Accordingly, inhibition of mTOR via temsirolimus or PI3K via LY-294002 hyperactivated in MVP-positive as compared to control transfected cells. Additionally, re-expression of MVP on subcutaneous and orthotopic tumour formation in SCID mice was tested.

Conclusions: MVP expression was an important determinant for glioblastoma cell survival and proliferation.

Tumor Resistance to Anti-angiogenic Therapies in Renal Cell Carcinoma Tumorgraft Mouse Models

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Background: Different types of tumors are currently treated with VEGF-targeting therapy as single therapy or in combination with chemotherapy, such as renal cell carcinoma (RCC). Nevertheless, clinical benefit of VEGF signalling inhibitors is short-lived and these therapies fail indeed to produce durable effects and to significantly modify the patient's long-term survival due to tumor adaptation and subsequent resistance to therapy.

Material and Methods: To investigate the mechanisms of resistance in a clinically relevant tumor, we have developed several Tumorgraft mouse models based on the orthotopic implantation of renal tumors derived from primary biopsies of human RCC tumors. We have evaluated the effects of VEGF signalling inhibitors of the murine VEGF2R (DC101) or the human VEGF ligand (Bevacizumab) after short and long term treatment, on the tumor microenvironment and the tumor growth of treated mice compared to the control. In the long-term therapy however we observed a tumor rebound due to the adaptation to treatment associated or not with an increase of vessel density. Ongoing experiments of molecular and immunohistological characterization will define the molecular mechanism of acquired resistance to the therapy.

Results: Short-term therapy with DC101 and Bevacizumab exerts an anti-angiogenic effect on RCC tumors that leads to a delay in tumor growth. Long-term therapy fails to control the tumor growth with an eventual rebound of tumor growth due to resistance to anti-angiogenic treatment. Results from new Tumorgraft RCC models based on primary human tumors could have relevant clinical implications in the understanding of the mechanisms involved in the acquisition of resistance to VEGF-pathway inhibition therapy in human patients.

Opposite Roles of Embryonic EMT-inducers in B-Raf-driven Melanocyte Transformation

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Originaly depicted as poor prognosis factors in light of their prometastatic potential, embryonic EMT inducers additionally behave as determinant drivers of the neoplastic transformation. Although numerous signals are likely to contribute to their aberrant reactivation during tumor progression, a growing body of evidence supports the hypothesis that their induction might be dictated by the initial mitogenic insult. We herein demonstrate that B-Raf activation, as recurrent genetic event in melanomas, induces a drastic upregulation of Zeb1 and Twist1 at the expense of Zeb2 and Snai2 in murine and human melanocyes. This reprogramming is determinant for B-Raf in promoting cell transformation, likely through the deregulation of cell differentiation/proliferation balance. Immunohistochemical expression analyses on a cohort of human nevi and melanomas support the hypothesis suggesting that EMT inducers either behave as oncogenes or tumor suppressor genes, according to the cellular context.

Serum Polymamines in Patients With Non-Hodgkin’s Lymphoma

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Background: The polymamines (PAs) putrescine (Put), histamine (His), spermidine (Spd) and spermine (Spm) are a group of naturally occurring compounds that are essentially involved in cell growth and differentiation, with especially elevated levels in fast growing tissues like cancer. They are studied as potential tumour markers. The non-Hodgkin lymphomas (NHLs) are a diverse group of blood cancers that include any kind of lymphoma except Hodgkin’s lymphoma, and vary significantly in their severity. As malignant diseases are in expansion, the aim of our work was to investigate polymamine levels, as dansylated derivatives, in serum of NHL patients, using LC/DAD technique, so we could apply it in clinical practice.

Material and Methods: This study involved sera of 12 patients with NHL (one with T type lymphoma, one with MALT type lymphoma, two with folliclar type lymphoma, and eight with diffusion large B celltype lymphoma), and of 13 healthy volunteers. We precipitated serum proteins using 0.4 M HClO4. At pH 6.0 we performed derivatization with dansyl chloride. 50 µl of prepared serum samples were injected into LC/DAD, in conditions of gradient elution, on C18 column. Commercially available Put, His, Spd and Spm were dissolved in different concentrations in ultra pure water; treated in the same way as serum samples and injected into LC for obtaining calibration curves by plotting the PKs peak area values against the respective concentrations of standards. The qualitative analysis was done using the method of retention time. Quantitative analysis was done using the method of external calibration.

Results: Retention times were 9.1 min for Put, 10.1 min for His, 13.2 min for Spd, and 15.4 min for Spm, respectively. Obtained data showed good linearity of calibration curves for Put, His, Spd, and Spm (R² = 1.0, R² = 1.0, R² = 0.99967, R² = 0.99985, respectively). It was noticed that concentrations of some special polymamines are very changed in some patients with NHL, compared with healthy subjects.

Conclusions: Concentration of polymamines in patients with NHL should be investigated depending on the type of NHL.

Embryonic Transcription Factors, MIRNAS and Mitogenic Stressors Network in Breast Tumorigenesis – De costing the Interactome

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Background: Reactivation of embryonic programs through Epithelial-to-Mesenchymal Transition (EMT) is associated with tumour initiation and progression endowing tumour cells with self-renewal potential and invasive properties. EMT is associated with a profound genetic reprogramming...
including reactivation of embryonic transcription factors and severe changes in miRNA expression. Cross-regulation between EMT-inducers and miRNAs was previously described like the Zeb1-miR-200 feed-back loop. Our goal is to determine whether specific expression networks between EMTs, miRNAs and mitogenic stresses exist in tumors, and represents a driving force towards transformation in breast tumorigenesis.

**Material and Methods:** We performed oncogenic cooperation assays in immortalized human mammary epithelial cells (HMEC-hTert) by using an EMT-inducer expression library in combination with various mitogenic proteins. As recently demonstrated in the laboratory, these combinations were efficient in transforming human cells, as assessed by soft-agar colony assay. EMT-inducer expression was next analyzed in a large number of colonies to evaluate a potential specificity of EMT-inducers according to the mitogenic insult. In parallel, we developed an in silico approach utilizing predictive algorithms to identify novel miRNAs targeting embryonic transcription factors.

**Results:** We found that Twist1 and Twist2 expression was specifically enriched in colonies generated in presence of a constitutively active form of b-catenin, whereas Snai2 expression was selected following PTEN depletion. The prediction of specificity of the identified combinations is currently being validated by bioinformatics tools. We additionally have identified novel miRNAs able to regulate EMT inducers and validated their down-regulation in a panel of basal B breast cancer cell lines. The interplay between miRNAs, mitogenic proteins and EMT inducers will be further examined by assessing their expression in a large cohort of Basal B/Claudin-low human breast tumours, a recently identified subtype displaying a mammmary stem cell state and EMT features.

**Conclusions:** Our results will widen our knowledge on the potential regulatory loops impacting on the epithelial/mesenchymal phenotype in order to evaluate their therapeutic value on drug resistance and stemness properties.

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**435 Invasion and Lymphatic Dissemination of Human Squamous Cell Carcinoma Xenografts in Three-dimensional Murine Microenvironment**

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**Introduction:** Squamous cell carcinoma (SCC) in tongue exhibits aggressive progression and early-stage lymphnode metastasis. The present study aimed at elucidating a putative linkage between carcinoma cell phenotypes, remodelings of microenvironment and malignant behavior in xenograft mouse model.

**Materials and Methods:** Three tongue SCC cell-lines (HSC2, OSC19, and OSC20) and two other SCC cell-lines (gingival-derived KOS2C and oral floor-derived HO-1u1) were used. Each cell-line grown in culture was transplanted into BALB/c nude mouse tongue. Tissue, and regional lymphnodes were dissected from the animals at periodical intervals after inoculation and then processed for preparation of paraffin-embedded serial sections. Growth of primary tumor and nodal metastasis were evaluated histopathologically and loco-regional alterations of carcinoma cell phenotypes were validated by bioinformatic tools and microdissection-coupled gene expression analysis. We also conducted histology-based 3D reconstruction using 50–100 serial sections to visualize simultaneously invasion modes of carcinoma foci and angiogenesis/lymphangiogenesis in the microenvironment.

**Results:** All five cell-lines gave rise to visible tumor masses (>2mm) in the tongue within 2–3 weeks, but individual cell-lines showed a diversity in proliferation activity assessed by Ki67(1+), intra- and peri-tumor densities of bloodlymphatic vessels, and EMT-like phenotypic alterations. Notably, all the tongue SCC cell-lines yielded metastatic loci at high frequency as early as 6 days after inoculation, whereas the non-tongue SCC cell-lines showed modest proliferation in the inoculation site and lower metastatic potential. 3D visualization of tumor architecture disclosed discrete invasion modes between SCC cell-lines, such as from massive growth with pushing border (OSCC1, OSC20) to branching (HSC2, KOS2C) and fingering invasion (HO-1u1). Although individual cell-lines showed loss of E-cadherin guiding invasion margin, in couple with upregulation of E-cadherin repressor ZEB1/2, vimentin expression varied markedly among tongue SCC cell-lines, namely null in OSC19, low in HSC2, and high in OSC20. These SCC phenotypes were carried over in metastatic nodal loci.

**Conclusion:** The present xenograft model is suitable to understanding of early lymphatic dissemination of tongue SCC. The results also support the theory that SCC invasion is multi-modal phenomena taking place in the three-dimensional microenvironment.

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**436 Evaluating of BRCA1 and BRCA2 Germ-line Mutations and BRCA Risk Assessment of Mutation Carriers in Turkish Breast Cancer Patients**

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**Background:** A large number of germ-line mutations in the BRCA1 and BRCA2 genes have been reported worldwide, but little is known about the role of these inherited susceptibility genes in breast cancer risk among Turkish women. Furthermore, the founder mutations of our society are still unknown. Therefore the data of breast cancer patients may be beneficial to improve our knowledge about specific BRCA1 and BRCA2 mutations observed in Turkish population and their frequency.

**Material and Methods:** Nineteen women who had early and/or a family history of breast cancer were included in the study. All tumor regions and exome exons of BRCA1 and BRCA2 genes were screened by heteroduplex analysis followed by direct sequencing of selected variants. These variations were evaluated in GVGD (http://agrgnd.iarc.fr), HSF (http://www.umdb.org/HSF) and ESEfinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3) web based programs to confirm whether these mutations affected Brca1and Brca2 proteins structure and splicing ability. In addition, BRCA2PRO software was used for risk analysis.

**Results:** Eleven different mutations were detected: two previously described truncating mutation (1643delC and 5382insNC) and one previously described missense mutation (32 T>C), 2 novel truncating mutations (3545delEA and 5248insST) and two novel missense mutations (4837 A>G and 5202 T>G) in BRCA1, and four previously reported missense mutations (1114 A>C, 5744 C>T, 7397 T>C and 8187 G>T) in BRCA2. These 11 different mutations were represented in 31.96 (31/97) percent of this study. Furthermore, the missense mutation (7397 T>C in BRCA1 gene was observed in high frequency (10.3%) in this group. The effect on Brca1 and Brca2 proteins structure and splicing ability of determined novel alterations were analyzed by using GVGD, HSF and ESEfinder programs. The results of BRCA2PRO software showed that the level of risk for both breast and ovarian cancer increased with age in women who carried the mutation. Statistical analysis of our findings and clinicopathological features of patients were performed using Mann-Whitney and Fisher’s Exact Tests by SPSS-16 web based program. There were significant differences in the tumor size and Ki67 expression between cases with or without the BRCA mutations (p = 0.035 and p = 0.014, respectively).

**Conclusions:** These findings contribute significantly to what currently is known about BRCA types and impact of germ-line BRCA1 and BRCA2 mutations in Turkish women. It is shown that determined novel mutations may have a role on breast carcinogenesis. The missense mutation (7397 T>C) in BRCA2 gene may have the founder effect on Turkish breast cancer patients. These observations may be of potential guiding management in BRCA1 or BRCA2 patients considering breast-conserving therapy.

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**437 FADD Deficiency Causes in Apoptosis and Necroptosis of Mouse Embryonic Fibroblasts**

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**Introduction:** Necrosis, like apoptosis, can be strictly regulated and that form of necrosis is called necroptosis. FADD (Fas-associated death domain) protein is a key molecule of extrinsic apoptotic pathway that transduces signal from the membrane death receptors to caspase 8, but it also plays a pivotal role in activation of cell death was investigated using knock-out mouse embryonic fibroblasts (FADD−/−) irradiated with different doses of UVB radiation. Cell viability was estimated using MTT test, caspase activity using commercial caspase kits and necroptosis using MTT test with necrostatin-1.

**Material and Methods:** The effect of FADD deficiency on cell survival and activation of cell death was investigated using knock-out mouse embryonic fibroblasts (FADD−/−) irradiated with different doses of UVB radiation. Cell viability was estimated using MTT test, caspase activity using commercial kits and detection of necroptosis using MTT test with necrostatin-1.

**Results and Discussion:** Results showed that FADD−/− fibroblasts have lower proliferation rate than wild-type cells as their viability was reduced in comparison to wild-type cells, following the exposure to UV radiation. Increased activation of caspases 3/7 and 8 was observed in comparison to wild-type cells, following the exposure to UV radiation at intensity range 100–600 J/m2. Increased activation of caspases 3/7 and 8 was detected in the irradiated FADD−/− fibroblasts and these cells did not have an increased viability in the presence of necrostatin-1 in comparison to the wild type. Caspase 8 activation was not detected in either cell type after the exposure to 300 J/m2 of UVB.

**Conclusion:** From results we can conclude that UVB radiation in FADD−/− fibroblasts causes stronger induction of apoptosis due to intrinsic pathway
**Function and Regulation of G-protein-coupled Receptor Kinase 2 (GRK2) in Tumor Progression of Stratified Epithelia**

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**Background:** G-protein-coupled receptor kinase 2 (GRK2) causes desensitization of GPCRs, such as chemokine receptors, turning down their signaling. It has been described that reduced GRK2 levels potentiate leukocyte chemotaxis, and emerging evidence suggests that GRK2 expression modulates epithelial cell migration in a cell-type and stimulus-dependent manner. Our main focus has been to study the function and regulation of GRK2 expression in tumors of stratified epithelia.

**Results:** Interestingly, we detect GRK2 expression in differentiated areas of these tumors but not in the invasive front areas. Moreover, there is a significant negative correlation between GRK2 expression and tumor malignancy and that could be useful in tumor prognosis. In this regard, overexpression of GRK2 in spindle-like cells induces an epithelial phenotype, and preliminary results indicated that reduced GRK2 levels increase migration of human keratinocytes. Moreover, the skin of GRK2 heterozygous mice displays an altered pattern of expression of genes involved in cell cycle and cell proliferation. We also find that in the skin of these animals there is a downregulation of miR-145, a tumor suppressor miRNA that directly targets several proteins involved in cell migration and inhibits proliferation.

**Conclusion:** Our results support a putative inhibitory role of GRK2 in tumoral progression in stratified epithelia.

**Evidence for the Induction of Autophagy by AntiRin2**

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**Introduction:** miRNA-21 has been described as being a pivotal microRNA in several cancers, including leukemia [1]. In fact, its expression has been associated with several cancer related cellular processes such as decrease in apoptosis [2], or increase in proliferation and invasion [3]. In previous work we have used antimiRs for miR-21 (chemically modified, single stranded nucleic acids designed to downregulate miR-21 expression) in order to sensitize chronic myeloid leukemia cells to cytotoxic drugs. This downregulation increased programmed cell death detected by TUNEL, but no PARP cleavage was observed by Western Blot, suggesting that antimiR-21 might be inducing a cell death mechanism other than apoptosis. We also had some evidence of autophagy (work submitted for publication). Based on these results, the aim of the present work was to confirm if antiRin2 induced autophagy in a chronic myeloid leukemia cell line (K562).

**Materials and Methods:** A chronic myeloid leukemia cell line (K562) was transfected with antiRin2 or antiRin2 or control anti-Rin2-48 hours after transfection, by RT-PCR. Expression of proteins related to autophagy (Beclin-1, Vps34 and LC3-3) was analysed, by Western Blot. Cells were incubated with monodansylcadaverine (MDC, a fluorescent marker for 1h, and then the autophagic vacuoles were visualized by transmission electron microscopy. The cellular ultrastructural morphology, particularly the presence of autophagosomes, was observed by transmission electron microscopy (TEM).

**Results:** It was confirmed that transfection of K562 cells with antiRin-2 caused downregulation of miR-21 expression, 24 hours later. This downregulation caused an increase in the expression levels of Beclin-1, Vps34 and LC3B-II, which have been associated with autophagy. Accordingly, fluorescent double-staining, labeling autophagic vacuoles, typical of MDC staining, were visualized. The presence of autophagic vacuoles in K562 cells was further confirmed 48h after antiRin2 transfection, by TEM.

**Conclusions:** AntiRin2 induced autophagy in K562 leukemic cells.

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**Reference(s)**

Material and Methods: We assessed the relevance of CD157 in tumour migration and dissemination by transfecting CD157 cDNA in CD157-negative NIH/OCV-CAR-3 and OV-90 cells expressing low CD157, or by knocking down CD157 expression in OV-90 cells. These cells were used as in vitro models to dissect the functional role of CD157 during crucial steps of ovarian cancer progression.

Results: Exogenous expression of CD157 in NIH/OCV-CAR-3 and overexpression in OV-90 cells demonstrated that high levels of CD157 induce morphological and functional modifications reminiscent of mesenchymal-like differentiation and promote tumour cell survival by inhibiting anoikis. The effects of CD157 overexpression on ovarian cancer cells phenotype translate into increased tumour cell motility, mesothelial invasion and matrix metalloproteinases activity. Conversely, knockdown of CD157 in OV-90 cells reverts the mesenchymal phenotype and reduces the migratory potential, implying a direct correlation between CD157 expression levels and tumour aggressiveness. Gene profile analysis highlighted 378 significantly deregulated genes representing the signature of CD157-overexpressing ovarian cancer cells. The analysis of these transcripts indicated that expression of CD157 strengthens a number of pro-metastatic biological processes (such as motility and invasiveness), and represses several biological processes counteracting tumour progression (such as apoptosis, cell death and response to stress).

Conclusions: Collectively, these findings support a role of CD157 in the control of ovarian cancer progression and motivate the existence of a direct correlation between the expression levels of CD157 and the adverse clinical outcome in patients.

CD157 is a Novel Prognostic Marker of Malignant Pleural Mesothelioma Involved in the Control of Tumor Aggressiveness

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Background: Malignant pleural mesothelioma (MPM) is an aggressive and therapy-resistant tumor arising from pleural mesothelial cells. Recently, we have demonstrated that CD157, a GPI-anchored NARead/ADP-ribosyl cyclase regulating leukocyte diapedesis, is expressed by epithelial ovarian cancer and peritoneal mesothelial cells, controls tumour cell migration and peritoneal invasion and is a marker of poor prognosis. Because ovarian cancer and MPM share many similarities in terms of aggressiveness and progression, we hypothesized that CD157 could be involved in the control of the invasive behavior of MPM. To address the clinical and biological significance of CD157 in MPM, we investigated (i) the expression of CD157 in surgical samples of MPM and its correlation to clinical-pathological features and outcome, and (ii) its functional effects in both native and engineered mesothelial-like cells.

Materials and Methods: CD157 expression was examined by immunohistochemistry in tissue sections from surgically resected MPM (n=81), quantified by histological score (H-score), categorized as 0/1/2 above or below the median value of 60, and compared with clinical parameters. The functional role of CD157 was investigated by transfecting CD157 in MPM cell lines that have been used to study tumor cell migration, invasion, proliferation, and mesothelioma with conventional invito cell-based assays.

Results: CD157 proved to be expressed in 85% of MPM analyzed and CD157-H-score was associated with outcome on follow up in patients with biphassic MPM (P=0.034, n=43). Cox regression showed that CD157-H-score and membrane localization were independent prognostic factors of poor prognosis in biphassic MPM. Moreover, CD157 proved to be expressed in selected cell lines andits expression in the CD157-negative MPM cell lines significantly enhanced tumor cell motility and invasiveness through extracellular matrix proteins, increased tumor cell proliferation, and induced resistance to chemotherapy.

Conclusions: These findings indicate that CD157 is expressed by the majority of MPM and its high level of expression and membrane localization are associated with a shorter survival in patients with biphassic MPM. Moreover, CD157 is involved in the control of migratory and invasive behavior of biphassic MPM and might represent a novel biomarker with potential clinical utility.
Distinct Roles of AKT Isoforms in Regulating Beta1-integrin Activity, Migration and Invasion in Prostate Cancer

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Background: AKT1/PKB kinases are the primary downstream mediators of PI3K signaling and they influence numerous cellular processes including survival, proliferation, metabolism and migration. The AKT family of kinases includes 3 members, AKT1, -2 and -3 which share a high degree of homology. Several studies have investigated the specific roles of individual AKT family members in cell migration and invasion in breast and ovarian cancer. Recently, interesting molecular details have been identified regarding the opposing functions of the AKT isoforms in cell migration. Integrins are transmembrane receptors which mediate cell adhesion to the extracellular matrix. Active integrins are known to be crucial for cell adhesion, migration and invasion and they are involved in cancer cell motility and metastasis. In this study, we have investigated the isoform-specific functions of AKT in prostate cancer.

Material and Methods: Different AKT isoforms were silenced from PC3 prostate cancer cells and b1-integrin activity from these cells was measured. Migration and invasion assays were performed with AKT silenced cells.

Results: A RNAi screen for integrin activity inhibitors performed in our laboratory identified AKT1 as an inhibitor of b1-integrin activity in prostate cancer. Validation experiments demonstrated that, unlike in breast cancer, both AKT1 and AKT2 function as negative regulators of cell migration and invasion in PC3 cells. Downregulation of AKT1 and -2, but not AKT3, induced activity of cell surface b1-integrins and enhanced adhesion, migration and invasion. Silencing of AKT1 and -2 also resulted in increased focal adhesion size. Immunofluorescence, Western blot and cell fractionation studies were used to assess p120ctn localization. In vitro functional assays were performed to measure cell migration and invasion capacity, and small GTPases activity was measured by G-elsa assays. Protein-protein interactions between cadherins and catenins were evaluated by in situ proximity-ligation assay.

Conclusion: This work presents a new cellular mechanism that explains why P-cadherin expression is associated with a poor patient survival in breast cancer, and opens new therapeutic approaches, using dasatinib, to treat these tumors.

Reference(s)

Regulation of the Metabolic Profile of Breast Cancer Cells by Hypoxia

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Background: One essential hallmark of tumour cells is the glycolytic phenotype, which can be an adaptive consequence of tumour hypoxic microenvironment. Hypoxia, a common feature of malignancy, promotes hypoxia-inducible factor 1 alpha (HIF-1α) stabilization, which regulates the expression of several glycolytic markers. It has been described that monocarboxylate transporters (MCTs) by transporting lactate are important in the maintenance of the glycolytic phenotype and intracellular pH homeostasis, contributing to acidic microenvironment. However, MCT regulation in the presence of hypoxia is not well understood and is sometimes controversial, especially in what concerns MCT1 regulation.

Aims: We intend to characterize the expression of MCTs and other glycolytic markers, under hypoxic conditions. We also aimed to evaluate the effect of hypoxia on cell metabolism, as well as to determine the sensitivity of human breast cancer cell lines to CHC (an MCT inhibitor).

Material and Methods: Hypoxia was induced by incubation of breast cancer cell lines (MDA-MB-468, MDA-MB-231, Hs578T and MCF7/7A) in a hypoxic chamber with <1% O2, Characterization of the expression of MCT1, MCT4 and
CD147 and other metabolic markers was performed by immunocytochemistry and Western blot. The effect of hypoxia on cellular metabolism was assessed through quantification of glucose consumption and lactate production and the effect of CHC on cell total biomass through the Sulfrohodamine B assay.

Results and Discussion: As expected, some glycolytic metabolic markers increased, namely GLUT-1, CAIX and LDH. In general, MCT1 expression increased (e.g. HS578T cells) or its cell location was altered. However, the increase of MCT4, which is described as highly induced under hypoxia, was not so evident. Curiously, the expression of the MCT chaperone CD73 appeared to decrease in some situations (e.g. MCF7/A21). As expected, the metabolic profile and sensitivity to CHC was also affected by hypoxic conditions.

Conclusion: In this study, we showed that hypoxia regulates the expression of MCTs, mainly MCT1, and also affects cell response to MCT inhibition. Further studies will be needed, however, these findings provide some evidence for the role of MCT regulation by hypoxia and for the importance of MCT1 in the glycolytic metabolism.

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454 LFA-1/ICAM-1 Interaction Plays a Key Role in the Metastatic Development of C26 Colon Carcinoma Cells to the Liver

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Lymphocyte Function Associated-1 (LFA-1) of an Intercellular Adhesion Molecule-1 (ICAM-1) ligand has been extensively reported. However, the role of this molecule in metastatic development and solid cancer is not very well understood. In previous studies, we reported this interaction to be needed for stimulation of Mannose Receptor (ManR) in liver sinusoidal endothelial cells (LSEC), which resulted in a decreased cytotoxicity potential of ISL towards C26 colon carcinoma cells. Addition of tumour cells with ICAM-1 increased tumour development in the liver. Thus, we aim to study the lack of this molecule during C26 colorectal cancer cell interaction with LSECs and its implications in the metastatic progression of colorectal cancer. To do so, C26 colorectal cancer cell line deficient in LFA-1 was used. Endocytosis of LSEC after co-culture was measured by endocytosis assay of DQ-Ovalbumin. The migratory potential, adhesion and proliferation capacity of C26 cells were analysed by modified-Boyden chamber assay and CFSE assay, respectively. The metastatic potential was quantified by using an experimental model of liver metastasis development of C26 in Balb/c mice. The lack of LFA-1 expression in C26 colon cancer cells turned these cells unable to stimulate ManR activity on LSECs. Additionally, the in vitro proliferation, migration and adhesion decreased in LFA-1 deficient C26 cell line compared to wild-type C26 cells. Along with these results, a decrease in the in vivo metastatic potential was observed. The immune system cell phenotype pattern was also modified in LFA-1 deficient C26 cell line bearing mice with respect to those injected with C26 parental cell lines. Thus, the LFA-1/ICAM-1 interaction accounts for some of the main metastatic advantages which confer C26 cells the ability to aggressively metastasize to the liver. Based in these results, the LFA-1/ICAM-1 interaction accounts as an important pathway for colorectal cancer cell to metastasize to the liver. Ongoing studies based using host-ICAM-1 siRNA in vivo would provide more insights about this pathway as therapeutic target in cancer therapies.

455 Epithelial-mesenchymal Transition of Small Cell Lung Carcinoma

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Background: Small cell lung cancer (SCLC) is a highly malignant and aggressively growing tumor. It is early metastasizing and prognosis of patients is poor. For the process of metastasis, cells need to change their morphologic pattern and develop invasive abilities. Cells are disbanding cell-cell connections, acquire migration capabilities and are showing spindle shape appearance. These processes are named epithelial-mesenchymal transition (EMT). We investigated EMT processes based on morphologic differences in SCLC cell line populations. Differences in EMT markers between adherent and floating SCLC subpopulations could be shown. We were also able to induce these phenotypic changes by BrdU treatment.

Material and Methods: We investigated the small cell lung cancer cell lines (NCI-H69, MOG-H69V, NCI-H62, NCI-H446, NCI-N392) and analyzed morphologic and epithelial marker on RNA and protein level, based on morphologic differences. Furthermore, we analyzed migration, invasion and chemoresistance. In order to simulate EMT processes, we induced morphologic changes by BrdU.

Results: The analyzed SCLC cell lines showed morphologic changes under treatment with 10 µM BrdU. After 10–14 days NCI-H62, NCI-H69 and NCI-N392 changed their appearance from floating clusters to adherent and spindle shaped cells. All adherent sub-cell lines showed similar upregulation of mesenchymal marker as Vimentin, Fibronectin and FSP1. Epithelial marker such as E-Cadherin and Zona-Occuludens were found to be downregulated. In line with EMT processes the migration and chemoresistance was significantly higher in the adherent sub-lines. Thus, adherence seems to go along with EMT and several malignant abilities.

Conclusion: We suggest that adherent subpopulations, which underwent EMT, show higher tumourigenic potential. This could be an interesting target for future therapy strategies, especially to overcome chemoresistance and to prevent relapse.

456 Involvement of Matrix Metalloproteinases in the Epithelial-mesenchymal Transition and Metastasis of Small-cell Lung Cancer

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Background: Small-cell lung cancer (SCLC) is characterised as highly aggressive and early metastasising tumour with initially high chemosensitivity followed by fast relapse with chemoresistance. The process of metastasis often includes epithelial-mesenchymal transition (EMT) processes. EMT is characterized by the loss of cell-cell contacts and by the acquisition of cell motility and mesenchymal markers. Moreover, tumor cell invasion depends on extracellular matrix degradation. This is mostly caused by increased secretion and/or activation of proteases like Matrix metalloproteinases (MMPs). Hence, we analyzed the link of EMT-processes and MMPs in SCLC cell lines.

Material and Methods: We analysed adherent variants and classic SCLC cell lines to investigate the link of EMT and MMP secretion. For this, we used DO™ Collagen, type IV (Invitrogen) degradation assay, MMP antibody arrays (Raybiotech), gelatin zymography and RT-PCR.

Results: Classic SCLC cell lines typically grow in floating aggregates. Adherent SCLC variants, after selection of sub-populations or after treatment with 5-bromo-2′-deoxyuridine, show a more mesenchymal morphology, accompanied by an upregulation of mesenchymal markers. The adherent variants show a higher extracellular proteolytic activity of DO™ Collagen, type IV (Invitrogen) degradation analysis performed with MMP antibody arrays revealed an increased secretion of MMPs, especially of MMP-9 and MMP-13. Zymography showed an increased activity of MMP-2 and MMP-9. RT-PCR analysis confirmed that MMP expression was upregulated in adherent variants.

Conclusion: In summary, adherent SCLC cells undergo an EMT, with subsequent enhanced secretion and activation of MMPs. Thus, we suggest that the interplay of EMT processes and MMP secretion might play a role in SCLC metastasis formation.

457 Evaluation of Expression of E-cadherin in Epithelial-mesenchymal Transition and Its Relationship With Cell Proliferation in Esophageal Squamous Cell Carcinoma

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Introduction: Esophageal squamous cell carcinoma (ESCC) has a poor prognosis mainly because it is usually in an advanced stage at the time of diagnosis. The epithelium-mesenchyme transition (EMT) is the main event in tumor progression and metastasis, when epithelial cells lose their original characteristics and acquire mesenchymal phenotype, indicated firstly by the loss of E-cadherin expression. This study aimed to elucidate whether tumor cells that have undergone EMT at the invasive front are in proliferative state.

Material and Method: Samples of 58 ESCC cases were double stained by immunohistochemistry for E-cadherin (BD Bioscience) and Ki67 (ROCHE) antibodies using the Automated System Ventana BenchMark XT (ROCHE). Immunohistochemical analysis was performed using Aperio ScanScope XT (APERIO) with Algorithm ColorDeconvolution.

In each case E-cadherin and Ki67 expression were evaluated in the tumor core and at the invasive front, separately. Positivity was divided into strong, moderate and weak positive according to intensity staining. For each case a score (range 0–300) was given based on the sum of the percentages of each positivity group (Score = % Weak+ % Moderate+ % Strong). Results and Discussion: Among the 58 E-cadherin on ECADC was 163.5 ± 22.4 (p < 0.0001). In 88% of the cases, E-cadherin expression was more frequent in the tumor core when compared to the invasive front, where the expression was...
found in 12% of the cases. However a moderate positive correlation (r = 0.646, p < 0.0001) was found between ECADF and ECAD expression.

Melanoma invasion at the invasive front (KI67F) was 9.6±1.9 % and in the tumor core (KI67C) 5.2±1.1 % (p=0.002). In 72% of the cases KI67 expression was higher at the invasive front than in the tumor core, and 28% showed Ki67 expression more frequently in the core. There was a regular positive correlation between KI67F and KI67C (r = 0.276, p = 0.036). Although significant, a regular positive correlation between ECADF and KI67C was observed (r = 0.341, p = 0.009), indicating that the E-cadherin loss by tumor cells in EMT at the invasive front did not imply an increase of proliferative status.

Conclusion: According to our findings, tumor cell at the invasive front did not show an increased proliferation rates as it loses the E-cadherin expression. A more complex mechanism in the dynamics of E-cadherin and KI67 expression could be acting during EMT. Further studies are necessary to the better understanding the complexity of this mechanism.

459 Down-modulation of MMP9 Contributes to the Inhibitory Effects of MicroRNA-146a on Melanoma Cell Invasiveness

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MicroRNAs (miRNAs), that are post-transcriptional regulators of a large portion of the entire genome, have been implicated in oncogenesis. We previously showed that the majority of melanoma cell lines express miRNA-146a levels lower than those of normal melanocytes. In this study we investigated whether miRNA-146a modulates melanoma cell invasiveness. Two cell lines with normal and two cell lines with low levels of miRNA-146a, respectively, were transfected with 50 nM of a dsRNA mimicking mature miRNA-146a (miRNA-146a/prec) or of a control dsRNA (dsRNA-CTRL) and analyzed for the ability to invade the extracellular matrix (ECM) 48 h after transfection. ECM invasion was initially evaluated under basal conditions or in response to human fibroblast conditioned medium. The ability to invade the ECM of miRNA-146a/prec-transfected cells was found to be lower (50-70% inhibition) than that of the corresponding dsRNA-CTRL-transfected cells, when the two cell lines under-expressing endogenous miRNA-146a were considered. In contrast, ectopic expression of miRNA-146a did not significantly affect invasiveness of the cell lines endowed with normal miRNA-146a levels. Further studies performed in one of the melanoma cell lines with reduced levels of miRNA-146a (i.e. WM-115), showed that ECM invasion in response to VEGF-A, but not cell adhesion on ECM, was also markedly impaired (70% inhibition) upon transfection with 50 nM miRNA-146a/prec. To get insight into the possible mechanisms underlying the effects of miRNA-146a on melanoma cell invasiveness, WM-115 cells were transfected with 50 nM miRNA-146a/prec or dsRNA-CTRL and analyzed, by real time RT-PCR, for the expression of a panel of 90 genes involved in cell adhesion, migration and invasion. Several genes resulted differentially expressed in miRNA-146a/prec-transfected cells with respect to control cells. Among the down-regulated genes, MMP9, coding for matrix metalloproteinase-9, was selected for further studies. Down-regulation of MMP9 secretion in miRNA-146a/prec-transfected WM-115 cells was confirmed by ELISA assays on cell culture supernatants. Moreover, we found that anti-MMP9 neutralizing antibodies were able to impair VEGF-A-induced ECM invasion. Our results suggest that down-modulation of MMP9 expression contributes to the inhibitory effects of miRNA-146a on melanoma cell invasiveness.

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200 levels in primary tumor-derived cell lines. We found miR-200 depletion promoted wound healing and cell migration towards serum. Transplantation of the sponge-expressing lines via tail vein led to in vivo formation of more invasive, metastatic tumors compared to controls. Previously, the miR-200 family has been shown to inhibit the TGF-β1 target gene and EMT mediator, Zeb1. We have identified another miR-200 target, Epidermal Growth Factor Receptor Substrate 8 (Eps8), which mediates actin based cell motility. In miR-200 low (M) cells, Eps8 is expressed three-fold higher than in corresponding miR-200 high (T) cells. Additionally, exogenous expression of Eps8 in miR-200 high, T cell lines promotes wound healing and cell migration, similar to miR-200 sponge expressing cells. Thus, our findings implicate the miR-200s as a class of metastasis regulators that modulate expression of genes functionally relevant in tumor cell dissemination and provide new targets for therapeutic intervention of late stage lung adenocarcinoma.

460 Direct Detection of TMPRSS2-ERG Rearrangements in Prostate Cancer by Padlock Probes

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Background: With the increasing number of translocations known to play a role in the genesis of solid tumors, there is a need for direct tissue localization of gene fusions in molecular pathology. Current methods for detection of TMPRSS2-ERG rearrangements provide qualitative and quantitative information on the rearrangement but no information on the expression of the different fusion variants and their site in situ localization. We here present the first technology for multiplex in situ detection of expressed fusion genes.

Materials and Methods: Padlock probes were designed for the most prevalent fusion transcripts (T1/E2, T1/E4, T1/E5, T2/E4 and T2/E5) with the ability to discriminate between them from each other. These wild-type and fusion-positive human prostate cancer cell lines VCaP and LNCaP, respectively, were used to test padlock specificity. After confirming the quality of the probes, the in situ padlock technique was applied on formalin-fixed paraffin-embedded (FFPE) human prostate cancer tissue sections. These tissue sections were analyzed for the expression of identified fusion TMPRSS2-ERG fusion transcripts.

Result and Discussion: We here demonstrate the establishment of multiplex in situ detection of fusion variants in FFPE human prostate cancer tissues by using the prevalent TMPRSS2-ERG translocations.

Several TMPRSS2-ERG fusion genes have to date been identified, and we here identify the expression of the most prevalent fusion transcripts (T1/E2, T1/E4, T1/E5, T2/E4 and T2/E5) by using fusion-specific padlock probes and rolling-circle amplification.

Conclusion: This novel in situ detection method holds great potential as a tool to investigate translocations in solid tumors and their role in tumor progression as possible biomarkers or prognostic markers.

461 MEK and SRC Inhibitors as a Combinatorial Approach to Melanoma Therapy

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Background: It has been widely shown that the vast majority of malignant melanomas have deregulated signalling in the RAS-RAF-MEK-ERK pathway. These findings have prompted a surge in development of inhibitors targeted to the component kinases of the cascade. In particular, the targeting of MEK is currently being tested in clinical trials. We have looked at the effect of two MEK inhibitors (AZD6244 and PD184352) on melanoma cell-lines in-vitro. As melanoma is a highly metastatic cancer, we have looked closely at the effects of these drugs on adherence and invasion. SRC kinases are known to have a central role in migration and invasion through their regulatory role in focal adhesion formation. A SRC inhibitor (AZD0530) was used in combination with MEK inhibitors to determine whether there was therapeutic potential to this approach.

Materials and Methods: Experiments were conducted using MEK inhibitors PD184352 and AZD6244, and SRC inhibitor AZD0530. Relative cell invasion through a collagen matrix was determined using an inverted invasion assay and melanoma cell lines. Relative adhesion formation was determined by allowing cells to adhere to a collagen substrate for 30–60 min. Gelatine zymography was used to assess MMP activity. MMP expression was shown with p4C2. Proliferation rate was assessed using the Click-IT EdU kit from Invitrogen.

Results and Discussion: SRC inhibition had little impact on cells entering S-phase in melanoma cell-lines but striking anti-invasive effects. This was confirmed by a significant loss in adherence to collagen substrate. However, we have found that whilst MEK inhibition can efficiently suppress tumour cell growth, it can also increase cell invasiveness. After treatment with MEK inhibitor, cells displayed an increase in integrin-mediated adhesion
and matrix-metalloproteinase-2 (MMP-2) expression. Together this induced a mesenchymal mode of invasion. As this is dependent on adhesion, we used a 3D lung carcinoma cell line (A549) to disrupt focal adhesions. This approach significantly reduced cell adherence and in turn effectively abolished MEK-MAP kinase pathway in melanoma.

Material and Methods: Evaluation of glycolytic metabolic markers (MCT1, MCT4, CD147, GLUT-1, HKII, LDH-V, PDK) and hypoxic markers (CAIX and HIF-1α) expression was performed in a series of 50 glioblastomas by immunohistochemistry. The immunohistochemical evaluation were performed taking into account the intensity of staining, as well as the cellular localization in peri-necrotic (hypoxic regions) and non peri-necrotic (normoxic regions) regions of the glioblastoma tissues. Results: Expression of MCTs, MCT chaperone CD147 and other metabolic markers increased significantly in hypoxic regions compared to normoxic regions, and the expression of GLUT-1, CAIX and HIF-1α was restricted to areas adjacent to necrosis. All metabolic markers and MCT isoforms evaluated correlated to CAIX and HIF-1α expressions, with exception of CD147 with HIF-1α. Importantly, a significant increase to plasma membrane expression of MCT1, CD147, GLUT-1 and CAIX was found in hypoxic regions. The same association was not found for MCT4 isoform since it presents a high heterogeneity with high levels of hypoxia and glycolysis, which for instance confer resistance to therapy. It is important to characterize the distribution of the glycolytic metabolic markers, particularly MCTs, in the response to hypoxia.

Background: Glioblastoma is the brain tumor with the highest prevalence and lethality, being important to identify new molecular therapeutic targets that could improve the life time of these patients. Hypoxia is a common feature in malignancy, particularly in glioblastomas, leading to hypoxia-inducible factor 1 alpha (HIF-1α) stabilization which induces an increased glycolytic metabolism, due to the upregulation of several metabolic proteins. The high glycolytic rates of tumor cells increase lactate production, which is transported to the microenvironment through monocarboxylate transporters (MCTs), important in the maintenance of physiological intracellular pH. Knowing that glioblastomas present a high heterogeneity with high levels of hypoxia and glycolysis, which for instance confer resistance to therapy, it is important to characterize the distribution of the glycolytic metabolic markers, particularly MCTs, in the response to hypoxia.

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Conclusion: Using MEK and SRC inhibitors together has a strong impact on melanoma cell proliferation and invasion.

#462 MCT1 Plasma Membrane Expression is Associated to Hypoxic Regions in Glioblastomas

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Background: Glioblastoma is the brain tumor with the highest prevalence and lethality, being important to identify new molecular therapeutic targets that could improve the life time of these patients. Hypoxia is a common feature in malignancy, particularly in glioblastomas, leading to hypoxia-inducible factor 1 alpha (HIF-1α) stabilization which induces an increased glycolytic metabolism, due to the upregulation of several metabolic proteins. The high glycolytic rates of tumor cells increase lactate production, which is transported to the microenvironment through monocarboxylate transporters (MCTs), important in the maintenance of physiological intracellular pH. Knowing that glioblastomas present a high heterogeneity with high levels of hypoxia and glycolysis, which for instance confer resistance to therapy, it is important to characterize the distribution of the glycolytic metabolic markers, particularly MCTs, in the response to hypoxia.

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Conclusion: Using MEK and SRC inhibitors together has a strong impact on melanoma cell proliferation and invasion.

#463 Constructing Therapeutic Strategies for Ewing Sarcoma by Systems Biology Approach

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Introduction: Ewing’s sarcoma is characterized by a chromosomal translocation between EWSR1 gene and one member of ETS family gene. In 85% of the patients, this leads to the expression of the chimeric EWS-FLI1 resulting from the课外段DNA translocation in a subset of Ewing tumor cell lines. This translocation results in the expression of a chimeric EWS-FLI1 protein which acts as an activator of the transcription factor ETS-1, and induces the expression of a variety of genes that are involved in proliferation, cell survival, and resistance to therapy. The goal of this study is to identify potential therapeutic targets that could inhibit the proliferation of Ewing tumor cell lines.

Material and Methods: The 273 genes were selected on the basis of expression profiles of multiple genomic data: transcriptome time series on cell lines, transcriptome from biopsies, EWS-FLI1 CHIP-Seq experiments on Ewing cell lines. The selection was performed by performing a gene expression profiling of these heterogeneous data, focusing on genes that are targeted by small molecules. 1092 siRNAs (4 siRNA/genes) were transfected into the inducible cell line (a or doxycycline conditions) as well as in the parental cell line in a high-throughput phenotyping platform.

Results: We produced a list of possible targets that modify proliferation and/or apoptosis in Ewing tumor cell lines. A subclass of these targets has an effect only when EWS-FLI1 is active. Further experimental investigation is in progress, around one pathway that comes out from this list of possible targets.

Conclusion: We produced an influence network of EWS-FLI1 signaling, including selected pathways from transcriptome data. We extended this influence network with new pathways identified in high-throughput experiments.

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Vascular Remodeling of Tumor Blood Vessels as a Result of Anti-angiogenic Therapy – the Role of Vascular Wall-resident Mesenchymal Stem Cells Within These Processes

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Introduction: Tumor vessels, which are characterized by resistance to anti-angiogenic therapy using bevacizumab, are characterized by an increase in vessel diameter and a normalization of vascular structures. This normalization is achieved by the recruitment and integration of mature pericytes in the vessel wall for capillaries as well as smooth muscle cells for larger vessels. The molecular and cellular changes associated with this process and its importance for tumor growth, metastasis and therapy resistance are still largely unknown. Here, we hypothesized that mesenchymal stem cells which reside in the vasculogenic zone of adult blood vessels are mobilized from their niche, e.g. by signals released from tumor cells and contribute to vascular remodeling of tumor blood vessels by differentiating into pericytes as well as smooth muscle cells.

Material and Methods: Vascular wall resident Mesenchymal stem cells express the (neural) stem cell marker nestin. We used the transgenic Nestin-GFP mouse expressing GFP under the regulatory elements of the nestin promoter in order to evaluate the role of vascular wall resident mesenchymal stem cells during vascular remodeling of tumor blood vessels under the influence of the VEGF antibody bevacizumab. Tumor cells (B16F10 melanoma) were subcutaneously transplanted into the flank of the mice. Tumor and surrounding tissues were removed and analyzed.

Results and Discussion: Here we identified GFP(+)sca1(+)CD44(+)CD34(−)CD45(−) cells within the adult arterial adventitia from Nestin-GFP mice with properties of multipotency. These cells exhibit typical mesenchymal stem cell characteristics including cell surface markers in immunostaining and flow cytometric analyses, and differentiation into adipocytes, chondrocytes and osteocytes under certain culture conditions. Co-localisation studies of B16F10 tumor xenografts using Nestin-GFP mice in combination with bevacizumab treatment showed that the GFP(+) multipotent stem cells are recruited to the tumor and differentiate to pericytes and smooth muscle cells which cover the wall of newly formed blood vessels. Co-implantation of isolated mesenchymal stem cells derived from Nestin-GFP aorta together with tumor cells confirmed the potential of these cells to differentiate into pericytes and smooth muscle cells resulting in vascular stabilization.

Conclusion: Our results suggest that Nestin-GFP mesenchymal stem cells are apparently involved directly in vascular remodelling processes in terms of vascular stabilization, serving as a local source for pericytes and smooth muscle cells to establish the vascular wall resident mesenchymal stem cells. Our results may have to be considered in future strategies for anti-angiogenic tumor therapy. In particular, the molecular analysis of vascular stabilization could yield new strategies for single target genes, which could reduce the rate of drug-resistant tumors.

Study of the Glioma Initiating Cells CD44high/ID1high in Glioblastoma

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Glioma Initiating Cells (GICs) are considered to be responsible of tumor initiation, propagation and recurrence. We recently demonstrated that a subpopulation within the glioma tumor mass, characterized by high levels of CD44 and ID1, have tumor initiating capacity. We found that TGF-β enhances the GICs self-renewal capacity through the induction of the cytokine LIF. We are now observing that LIF is induced by TGF-β through the transcription factor Runx1. The knockdown of Runx1 in GBM-derived neurospheres results in a decrease in the TGF-β-LIF-mediated induction of LIF. Importantly, we observed the binding of Runx1 in the LIF promoter region in a ChIP assay. Runx1 is also important to maintain the GIC subpopulation, as a knockdown of Runx1 in patient-derived neurospheres decreases in CD44high/ID1high GICs. Moreover, we observed that GICs CD44high/ID1high are located in the proximity of tumor blood vessels in a perivascular niche. Cerebral endothelial cells are important to maintain the GICs niche by secreting specific growth factors such as TGF-β which is important to enhance the tumor initiation capacity of GICs.

It is known that GICs are responsible for tumor recurrence and resistance to conventional therapies. We observed that CD44high/ID1high GICs are resistant to radiotherapy in vitro and in vivo. By treating the cells with a specific TGF-βRII inhibitor we can overcome this radioresistance of GICs, indicating that the combination of TGF-βRII inhibitor and conventional therapies can be beneficial to improve GBM treatment.
Role of Monocarboxylate Transporters (MCTs) in the Regulation of the Metabolic Profile of Cervical Cancer Cells by Hypoxia

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Aims: To investigate the expression of MCT1 and MCT4 and their possible association with a different chaperone. Consistent with the decrease in MCT1 expression, the sensitivity to the pan-caspase inhibitor. Bax and Bcl-2 protein expression were analysed by western blotting. The activity of SE and crocin against the growth of prostate cancer cells in vitro was examined in 22rv1 cells xenograft in male nude mice.

Materials and Methods: Saffron extract (SE) was prepared by maceration with 75% ethanol for 72h at room temperature (1g/20ml). HPLC was employed to purify and characterize crocin from saffron. Cell viability after treatment with different concentration of SE and crocin was quantified by MTT assay. Apoptotic cells were determined using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak). Role of caspases was studied using the pan-caspase inhibitor. Bax and Bcl-2 protein expression were analysed by western blotting. The activity of SE and crocin against the growth of prostate cancer cells in vitro was examined in 22rv1 cells xenograft in male nude mice. Experiment was designed with the treatment regimen of Saffron extract or crocin per os (400mg/Kg and 200mg/Kg body weight respectively) for 28 days.

Results and Discussion: In vitro studies demonstrated that SE or crocin influenced cell cycle and induced apoptosis in a time- and concentration-dependent manner with IC50 values ranging between 0.4 and 4mg/ml for SE and between 0.26 and 0.95mM/ml for crocin. Normal and nonmalignant cells were not affected. Flow cytometry profiles revealed that most cells were arrested at G0/G1 phase with a significant presence of apoptotic cells. Western blotting analysis revealed that the expression of Bcl-2 was strikingly downregulated, while Bax was upregulated. Analysis of caspase activity indicated a caspase-dependent pathway with involvement of caspase-9 activation, suggesting an intrinsic pathway. In vivo xenograft growth inhibition of the established 22rv1 tumors by SE and crocin was observed. At the end of experiments a significant reduction in solid tumor volume was found in crocin treated mice when compared with control animals (56.8% vs control, 621±67 vs 1438±167 mg, p<0.001), whereas a light reduction of tumor mass was observed with SE (31.3% vs control, 988±147 mg [mean±SD] vs 1438±167 mg, p=0.063). In crocin treated mice a significant reduction of angiogenesis was also observed.

Conclusion: Taken together, these findings suggest that crocin possesses a strong antitumor activity against prostate cancer and has potential for use in combination with other anti-neoplastic agents in the treatment of locally aggressive prostate cancer. Further studies in this field should be performed.

Identification of a Role for Anti-apoptotic Bcl-xL in Breast Cancer

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Bcl-2 homologues such as Bcl-xL are major regulators of apoptosis that are frequently overexpressed in cancer cells. Their activity is understood to contribute to tumor progression by allowing cancer cells to survive to many death stimuli. Some Bcl-2 homologues may also exert additional oncogenic properties. Bcl-xL overexpression, in particular, is correlated with high tumour grade and poor outcome. The regulation of Bcl-xL is complex and involves multiple post-transcriptional mechanisms.

Conclusion: PPARD may have a tumor suppressive activity in CaP. Although more studies are needed (in vivo and in vitro) to decipher the role of FAO and PPARD in the biology of CaP, our studies have inherent therapeutic implications since PPARD and FAO can be pharmacologically modulated.

Saffron Extract and its Major Constituent Crocin Selectively Inhibit the Growth of Human Prostate Cancer Cell in Vitro and in Vivo

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Introduction: Crocus sativus commonly known as saffron is the raw material for one the most expensive spice in the world and is known for anti-cancer properties. The mechanism of saffron-induced cytotoxicity in tumor cells has not been adequately explored. In this study, we investigated the antitumor effects of Saffron extract (SE) and its major constituent crocin in malignant and nonmalignant prostate cancer cell lines.

Materials and Methods: Saffron extract (SE) was prepared by maceration with 75% ethanol for 72h at room temperature (1g/20ml). HPLC was employed to purify and characterize crocin from saffron. Cell viability after treatment with different concentration of SE and crocin was quantified by MTT assay. Apoptotic cells were determined using PI staining of DNA fragmentation by flow cytometry (sub-G1 peak). Role of caspases was studied using the pan-caspase inhibitor. Bax and Bcl-2 protein expression were analysed by western blotting. The activity of SE and crocin against the growth of prostate cancer cells in vitro was examined in 22rv1 cells xenograft in male nude mice. Experiment was designed with the treatment regimen of Saffron extract or crocin per os (400mg/Kg and 200mg/Kg body weight respectively) for 28 days.

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Conclusion: Taken together, these findings suggest that crocin possesses a strong antitumor activity against prostate cancer and has potential for use in combination with other anti-neoplastic agents in the treatment of locally aggressive prostate cancer. Further studies in this field should be performed.

Tumor Suppressive Activity of PPARD in Prostate Cancer

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Introduction: Cancer cells present an unusual utilization of glucose, favoring lactate production from pyruvate instead of directing it to oxidative phosphorylation, even in the presence of oxygen (Warburg effect). Despite the intensive work on glucose metabolism, the contribution of fatty acid oxidation (FAO)-related metabolic reprogramming and pathogenesis and progression remains obscure. A key modulator of this pathway, PPARD, has been recently implicated in other cancers, but its contribution to prostate cancer (CaP) and metastatic breast cancer is not yet understood.

Materials and Methods: We have used available databases as well as microarray information from CaP or control patient's biopsies to evaluate FAO regulating gene expression. By real time PCR analysis we have analyzed the expression of PPARD and FAO related genes on mouse prostate tissues (3 days and 6 months of age) in combination with other antineoplastic agents in the treatment of locally aggressive prostate cancer. Further studies in this field should be performed.
Hexavalent Chromium, Cancer Stem Cells and Dedifferentiation – a View to a Kill!

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Introduction: Despite many endeavors, all cancer theories have failed to explain how primary tumors evolve to metastasis, and cancer is still a paradox affecting millions of people. Hexavalent chromium (Cr(VI))-induced lung tumors are a particularly misunderstood subset of lung cancers, with yet no specific therapy and or diagnostic procedure delineated. Consequently, while the atmospheric levels of Cr(VI) are continuing to increase, people are dying of cancer and there is an urgent need to better understand this pathology. It has recently been proposed that the heterogeneous nature of cancer stem cells (CSCs) have been observed to enrich in Cancer Initiating Cells (CIC). Notably, we described enhanced enrichment in CIC by measuring mammosphere formation in low adherence conditions.

Materials and Methods: Using standard cell culture procedures and in vivo mice experiments, four cell lines (Con1, Ren2G2, DRRenG2 and DDRenG2) were established out of the immortalized human bronchial epithelial cell line, BEAS-2B. Cells’ subsequent characterization involved both classical and molecular cytogenetic techniques, standard flow cytometry studies, microsatellite analysis, metabolic studies with [13C]fluoro-2-deoxyglucose ([13C]FDG) and nuclear magnetic resonance spectroscopy (NMR). Sphere-formation assay was carried out in all the cell lines to look for CSCs. Results and Discussion: BEAS-2B cells culture at low density in the presence of 5 x 10−5 M Cr(VI) gave rise to the malignant RenG2 cell line. Successive rounds of injection in athymic mice allowed the attainment of the DRRenG2 and DDRenG2 derivative cell lines. As NMR studies revealed a more glycolytic phenotype for the derivative cell lines, we hypothesized that CSCs may have played a role in Cr(VI)-induced malignant transformation. To test this hypothesis, sphere-formation assay was carried out in all the cell lines, being only positive for the derivative ones, suggesting that a process of cellular dedifferentiation drove the emergence of CSC-like sub-populations. Flow-cytometry, western blot, as well as [13C]FDG studies confirmed the stem nature of the isolated cells and allowed to trace the cellular lineages along the malignization process. Genomic arrays further revealed a wide range of chromosomal imbalances that, along with the aberrant karyotype of the malignant cell lines, may justify some of their features.

Conclusions: The increase in malignant potential of RenG2-derivatized cell lines was a consequence of a process of cellular dedifferentiation. This process resulted in the emergence of a CSC-like population within the derivative cell lines which boosts their aggressiveness and resistance.

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ICAT is a Novel PTF1A Interactor That Regulates Acinar Differentiation and Displays Altered Expression in Pancreatic Ductal Tumors

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Introduction: The Pancreas Transcription Factor 1 (PTF1) complex is the major driver of acinar cell differentiation and acinar cells are the most abundant cell type in the pancreas, responsible for the production of digestive enzymes. In the adult pancreas, PTF1 contains two pancreas-restricted transcription factors: PTF1A – a BHLL protein – and RBPJ. This complex recruits pCaf, which acetylates PTF1A and enhances its transcriptional activity. Recent data using genetic mouse models imply acinar cells in the development of pancreatic ductal adenocarcinoma (PDA).

Material and Method: We performed a two-hybrid screening assay with two different libraries to identify novel PTF1A partners that might modulate its function. We validated the new interactions performed, we performed competitive overexpression and GST pull down experiments. We analyzed the effects of ICAT overexpression in cultured cells on cell differentiation. Finally, we examined ICAT expression in human PDA using immunohistochemistry.

Results: We identified ICAT (inhibitor of beta-catenin and Tcf4) as a novel PTF1A interactor. ICAT was previously reported as a b-catenin interacting protein regulating Wnt pathway and proliferation. We validated and mapped the ICAT-PTF1A interaction in vitro and in vivo. Moreover, we demonstrated that upon its overexpression in immortalized acinar tumor cells – ICAT negatively regulated PTF1 transcriptional activity in vitro and in vivo. This effect was independent of b-catenin and was mediated by direct binding to PTF1A and displacement of pCaf. ICAT also modulated the expression of Pdx1 and Sox9 in immortalized acinar cells. The repressive effects on the acinar programme suggest a role in ductal differentiation. In normal pancreas tissue, ICAT is expressed in all epithelial cell types: acinar and islet cells displayed exclusively nuclear staining whereas ductal cells showed only cytoplasmic staining. By contrast, exclusive nuclear staining was observed in 46% of PDA (n = 93); this staining pattern was associated with poor survival in a multivariable model (p = 0.001).

Conclusion: ICAT is a new regulator of acinar differentiation and it does so through a novel, Wnt pathway-independent, mechanism. Further work is required to establish its contribution to PDA progression and the mechanisms involved therein.

Determiner of HER2 Gene Status and Chromosome 17 Polysomy in Penile Carcinoma and Association With HER2 Protein Levels

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Background: Squamous cell carcinoma of the penis is a serious health problem in undeveloped countries. HER-2 gene is located at 17q21-q22 and codes for a transmembrane growth factor receptor whose overexpression associates with aggressive tumor growth and poor prognosis, being, therefore, a target for anti-cancer therapies. We evaluated HER-2 gene expression and chromosome 17 status in penile carcinomas.

Material and Methods: Immunohistochemistry (IHC) against HER2 was carried out in 195 penile carcinoma samples selected from the files of ACCamargo Hospital, Brazil, using the monoclonal CB11 antibody (Novocastra™), which recognizes specifically HER2 intracellular domain. One hundred five of those cases were spotted in a tissue microarray (TMA) block and were submitted to dual color fluorescent in vitro hybridization (FISH) using flourescently-labeled probes for HER2 locus and for chromosome 17 centromere (Dako™). Cases showing two signals of each probe were considered non-altered, those showing more than two signals of each probe were considered polysomic and those showing more EGFR signals compared to centromere signals were considered amplified.

Results: None of the cases showed membrane staining for HER2 by IHC. However, a clear and strong cytoplasmatic staining was observed in 28 cases (14.9%). The staining was intense and did not represent background or artifact. Univariate analyses showed that overexpression associated with higher histologic grade (p < 0.001) and lower overall survival (p = 0.007). Multivariate analyses showed cytoplasmatic HER2
as an independent risk factor for death (RR = 2.966; 95% CI [1.6−5.2]; p < 0.001). FISH analysis revealed a great 55 out of 60 cases without gene number alterations (91.7%), and five cases (8.3%) presenting polymorphism of chromosome 17. No amplification of HER2 gene was observed in this series. Four cases from the polysomy group (80%) were negative in IHC. Only one case from this group (20%) showed cytoplasmic expression of the marker.

**Conclusions:** Although no membrane staining was seen in penic SCC samples, presence of HER2 in cytoplasm is an important poor prognostic indicator in this neoplasm. Alterations other than gene number may explain this intriguing immunohistochemical profile, such as mutations analysis. Due to the lack of a 5-hydroxymethylcytosine HCC827 profile in the clinical management of penile carcinoma patients, more investigation regarding the biological function of this marker is necessary.

**479** Role of the Wnt/b-catenin in Thyroid Cancer

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Thyroid cancer is one of the most frequent endocrine tumors. In malignant tumors, mutations in b-catenin gene (bcat), lead to the constitutive activation of the Wnt/bcat pathway, promoting tumoral growth. This activation is characterized by cytoplasmatic-nuclear localization of bcatenin. Increasing evidences have shown the involvement of the Wnt/b-catenin pathway in earliest stages of thyroid tumoral progression.

The aim of this work was characterized the activation of the Wnt/b-catenin pathway in thyroid tumoral cells carrying RAS (HNNK), BRAF and PI3KCa mutation, the most frequent in thyroid cancer initiation.

**Methods:** Cell lines: Differentiated thyroid cancer cells conditionally expressing HRAS or BRAF oncogenes; human thyroid tumoral cells line, carrying RAS, BRAF and PI3KCa mutations. Evaluation of the Wnt/bcat pathway analyzing: levels of inhibited phospho-6Ser-GSK3b; cellular sub-localization of bcatenin; transcriptional activity of the bcat/TCF-complex (TOP/FOP-Luc-reporter-system). Knockdown bcat expression: lentiviral shRNA. BrdU assay.

**Results:** HRAS, but not BRAF oncogen, induce bcat localization increasing bcat-dependent transcriptional activity. In parallel there is an increase of GSK3b phosphorylation in the inhibitory Serine9 when mutated HRAS was expressed. We also found activation of the Wnt/bcatenin pathway in several human tumoral cells carrying RAS and PI3KCa mutations. The knock down of bcat expression in these cell lines leads to a dramatic reduction in cell proliferation, that is due to an induction of senescence. Preliminary results show that there is a reduction in tumoral formation in nude mice in the knock down cells in compare with the control cells.

**Conclusions:** Our results indicate a role of Wnt/bcat pathway in the proliferation of Thyroid cancer cells.

**480** Modulation of Oxidative Stress by Twist Oncoproteins

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**Introduction:** Twist proteins are HLH transcription factors essential for proper embryonic development and over-expressed in many human tumours. Among their oncogenic activities, induction of epithelial to mesenchymal transition leads to increased invasion and might confer to an epithelial cell a cancer stem cell phenotype. In addition, Twist factors override two oncogene-induced failsafe programs: senescence and apoptosis, thereby promoting the malignant conversion.

Reactive oxygen species (ROS) are by-products of incomplete reduction of oxygen and are generated in all aerobic cells. While required for different physiological processes, ROS are also important mediators of apoptosis, senescence and motility and can produce cell damage and are tightly linked to disease, especially to cancer. Because Twist factors and ROS impact on several common cellular processes, we investigated whether these oncogenes could control their different oncogenic activities by modulating reactive oxygen species.

**Material and Methods:** In this study several primary and immortalised cell types were used. Expression of genes of interest was modulated by silencing or mRNA degradation using siRNA or shRNA or retroviral expression. Expression levels were analyzed by immunoblotting or Real-Time PCR. ROS levels were assessed by DHE and CM-H2DCFDA staining and subsequent FACS analysis. Apoptosis was measured by Annexin V-Cy3 labelling followed by FACS analysis.

**Results and Discussion:** We first generate cell lines expressing either Twist1 or Twist2 and measured their intracellular ROS levels. We found that both factors significantly decrease production of reactive oxygen species. This result was confirmed in different cell types. We next knocked-down Twist activity using RNAi and showed that this induces an increase of ROS. We concluded that both Twist genes display an antioxidant activity. We next investigated whether this new activity could be linked to Twist oncogenic properties and found that protection from Myc induced apoptosis was dependent on ROS inhibition. Finally, we explored the molecular mechanisms of ROS regulation by Twist.

**Conclusion:** Our data unveil a new activity of Twist that might have important implications for its functions in both physiological and patho-physiological settings.

**479** The Impact of EGFR on Notch Protein Levels in Non-Small Cell Lung Cancer

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**Introduction:** The Notch signaling pathway is evolutionary conserved and has crucial roles in the development and maintenance of embryonic and adult tissues. Recently, Notch has attracted the interest of many researchers regarding its role in cancer. In all cases, it seems that Notch pathway functions are highly cell-type dependent. Breast cancers are often described as having EGFR amplification and over-expression.

Our data unveil a new activity of Twist that might have important implications for its functions in both physiological and patho-physiological settings.

**480** Heterogeneity Within the Cancer Stem Cell Population of the Triple-negative Breast Cancer Subtype

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**Background:** Breast cancer stem cells (CSCs) are thought to be responsible for tumorigenic capacity and chemoresistant potential of breast cancers, thus their elimination are crucial. Breast CSCs are often described as having a CD44+/CD24low− profile, however little is known about the functional heterogeneity within this population at single cell level.

**Material and Methods:** Single cell clones exhibiting CD44+/CD24low−/CD49f+ phenotypes were isolated from a triple-negative breast cancer cell line and evaluated due to their CD24 profile, epithelial- and mesenchymal protein expression and morphology using flow cytometry and immunocytochemistry. CSC characteristics, such as the ability to establish tumor in immune deficient mice, and self-renewal in vitro and in vivo were also tested.

**Results:** We found that the single cell clones represented by an epithelial morphology express epithelial markers and exhibit a CD44+/CD24low−/CD49f+ phenotype. Single cell clones represented by mesenchymal or mammosphere morphology were shown to express mesenchymal markers and to exhibit a CD44+/CD24low− phenotype.

**Conclusion:** We found that the single cell clones represented by an epithelial morphology exhibited epithelial markers and exhibit a CD44+/CD24low−/CD49f+ phenotype. Single cell clones represented by mesenchymal or mammosphere morphology were shown to express mesenchymal markers and to exhibit a CD44+/CD24low− phenotype. These results suggest that the CD24 levels in the CD44+/CD24low− subpopulations are correlated with cell morphology. However, we found that the single cell clones exhibited a mammosphere formation, since both the epithelial-like CD24+ and mesenchymal-like CD24− subpopulations include single cell clones exhibiting the ability to form mammospheres. The CD44+/CD24low− cells exhibited a mammosphere formation, since both the epithelial-like CD24+ and mesenchymal-like CD24− subpopulations include single cell clones exhibiting the ability to form mammospheres. The CD44+/CD24low− cells exhibited a mammosphere formation, since both the epithelial-like CD24+ and mesenchymal-like CD24− subpopulations include single cell clones exhibiting the ability to form mammospheres.
Acid-extruding Proteins as Potential Novel Targets in Human Breast Cancer

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Introduction: A fundamental property of solid tumors is a markedly altered pH-profile compared to normal tissues, with acidic extracellular, and often alkaline intracellular pH (pH). This at least in part reflects the increased glycolytic metabolism of tumor cells, necessitating increased acid extrusion to maintain survival. Acid extruding transporters are therefore potentially interesting targets in cancer. The aim of the studies presented here was to explore the roles and regulation of the net acid extruding transporters, the Na+/H+ exchanger NHE1 and the Na+,K+,Cl− cotransporter NBCn1, in human breast cancer.

Materials and Methods: A combination of cell biological and molecular biology approaches were employed, including immunoblotting, immunofluorescence, siRNA mediated knockdown, pH-sensitive fluorescent probes, adhesion and migration assays, as well as immunohistochemistry on patient tissue samples.

Results and Discussion: Expression of a constitutively active, truncated form of the Erbb2/HER2 receptor (ΔErbb2) greatly increased pH regulatory capacity in MCF-7 breast cancer cells, in a manner mediated by NHE1 and NBCn1 [1]. After ΔErbb2 expression or stimulation of wild-type ErbB receptors, NBCn1 was strongly upregulated at the mRNA and protein levels, and NHE1 was phosphorylated at Ser197, a known target for the 90 kDa Ribosomal S6-Kinase (p90RSK). In MCF-7 cells, NHE1 was strongly expressed in invadopodial rosettes, colocalizing with the invadopodial markers cortactin, F-actin and phospho-Tyr1465 Src, as well as with its known binding partner, ezrin and radixin [2]. NHE1 inhibition or siRNA-mediated NHE1 knockdown potently sensitized ΔErbb2-MCF-7 cells to cisplatin-induced apoptosis, whereas it enhanced their adhesion and 2D migration on collagen-I [1]. Finally, using matched sets of patient tissue we show that NHE1 and NBCn1 are upregulated in primary breast carcinomas and lymph node metastases compared to normal breast tissue, and we demonstrate that Na+, K+,Cotransport is a major determinant of pH regulation in freshly dissected human breast tumors [3].

Conclusion: Expression and regulation of the two major pH regulatory ion transporters NHE1 and NBCn1 are altered in human breast cancer, resulting in altered pH regulation. NBCn1 expression is strongly sensitive to ErbB receptor activity, and NHE1 regulates chemotherapy sensitivity and cell motility. This suggests that NHE1 and NBCn1 are potential targets in breast cancer diagnosis and/or treatment.

Reference(s)

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A Novel Metabolic Anti-cancer Therapy – Targeting Liver Tumors Through Mitochondrial Damage

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Introduction: One of the basis of modern oncology resides on the metabolic alteration of cancer cells, that explicates an increase of glycolytic pathway, the so called “Warburg Effect”. Glycolysis can be seen as a cancer anti-stress response to allow tumor dissemination protecting cells from ROS-induced senescence and apoptosis. Although the multi kinase inhibitor Sorafenib specifically targets tumor cells harboring the V600E mutation of B-RAF, additional mechanisms likely account for the elevated anticancer activity of the drug in tumors lacking this mutation, and for the emergence of chemoresistance.

Although the present study analyzes the in vivo effect of the multi kinase inhibitor Sorafenib (SFB) was investigated, alone and in combination with the glycolytic inhibitor 2-deoxyglucose (2dg), in a liver tumor model, the LCSC-2 (Liver Cancer Stem Cells) hypothesis is proposed. In particular we investigated the mitochondrial action of SFB.

Material and Methods: Drug toxicity was evaluated through Propidium Iodide (PI) assay. Gene expression was investigated by microarray analysis. Protein expression was evaluated by western blotting. Intracellular ATP level was assessed by chemiluminescence. Mitochondrial action was assessed by Respirometric Analysis. Mitochondrial potential was measured with the JC-1 probe.

Results and Discussion: We found that in rat hepatocarcinocarcinoma (LCSC-2) cells exposure to Sorafenib was not paralleled by significant inhibition of ERK phosphorylation, but, instead, elicited a raise of intracellular reactive oxygen species and the Ser172 phosphorylation of the AMP-activated protein kinase (AMPK), two events consistent with mitochondrial dysfunction. Accordingly, SFB led to a substantial reduction of oxygen consumption, cellular ATP level and mitochondrial transmembrane potential, and isolated mitochondria from rat liver were depolarized by the drug in vitro, indicating a direct effect of SFB on the organelle. Interestingly, in keeping with its inhibitory action on mitochondrial respiration, Sorafenib killed much more efficiently LCSC-2 cells, as well as mouse B16F10 melanoma and 293T human kidney carcinoma cells when associated with the glycolysis inhibitor 2-deoxyglucose.

Conclusions: Taken together our preliminary results identify in mitochondrial damage and generation of ROS a novel modality of cytotoxicity by Sorafenib; moreover, the synergistic action of SFB plus 2DG outline a novel combined therapy to eradicate liver tumors.

Protection Against ER Stress in the Tumor Microenvironment by Loss of P19ARF

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Cellular stress occurs during tumorigenesis due to a number of factors including an increase in reactive oxygen species, restricted nutrients and low O2 levels or hypoxia. More recently, endoplasmic reticulum (ER) stress has emerged as a major player in tumor pathology as a consequence of hypoxia, among other triggers. Although cells in the tumor microenvironment are considered to be more epigenetically stable than the tumor cells, they are exposed to the same cellular stresses as tumor cells. While the impact of these stresses on tumor cells has been well-studied, the effects on other cells in the tumor microenvironment remains to be elucidated. Here, we examine the effect of hypoxia-induced ER stress in the tumor vasculature and demonstrate activation of the Unfolded Protein Response (UPR) in the endothelium. The UPR is generally thought to be cytotoxic allowing cells to recover from stress through activation of the PERK pathway which globally halts translation through phosphorylation of the eukaryotic translation initiation factor 2α (eIF2α). However, after prolonged or excessive levels of stress, the UPR will trigger apoptosis. Here we show that the tumor suppressor p19ARF is activated in endothelial cells subjected to hypoxia or pharmacologically-induced ER stress leading to endothelial cell cycle arrest via p53-independent mechanisms. p19ARF has been well characterized to be induced by oncogenic activation in tumor cells and stabilizes p53 via sequestration of Mdm2 in the nucleolus thus activating a tumor suppressive response. Our data shows that transactivation of p19ARF in endothelial cells occurs by E2F1 which is induced during ER stress and that ER stress-mediated activation of p19ARF leads to inhibition of ribosomal RNA processing in the nucleolus, leading to cell cycle arrest and/or apoptosis. Further, loss of p19ARF in endothelial cells confers resistance to ER stress-induced cell cycle arrest and apoptosis resulting in endothelial cell proliferation regardless of p53 status. Taken together, our data suggests that there is a selective pressure for endothelial cells in the tumor microenvironment that have lost p19ARF expression. p19ARF−/− endothelial cells are able to survive and proliferate in the presence of ER stresses, leading to increased angiogenesis and ultimately promoting tumor growth. Therefore, ER stress represents a novel inducer of p19ARF and could provide targets for anti-angiogenic therapy independent of VEGF inhibition.

Distinct Recycling of Active and Inactive β1 Integrins

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Background: Cancer cell migration is critically coordinated by trafficking of plasma membrane receptors. Integrins are a large family of heterodimeric cell surface adhesion receptors consisting of 24 non-covalently associated α and β subunits. Integrins bind to extracellular matrix ligands such as collagen and fibronectin. Integrin-containing adhesions allow cells to exert adhesion forces between different parts of the cell body, which is a prerequisite for
cell shape changes and cell motility. Integrins undergo constant endo/exocytic shuttling to facilitate the dynamic regulation of cell adhesion. Integrin activity lowers the components of the extracellular matrix is regulated by the ability of these receptors to switch between active and inactive conformations. Also, several cellular signalling pathways have been described in the regulation of integrin activity under different conditions. However, the interrelationship between integrin activity conformation and their endocytic fate have remained incompletely understood. Here, we have investigated the endocytic trafficking of active and inactive β1 integrins in cancer cells.

**Materials and Methods:** We use novel antibody-based methods and fluorescent plate-reader to study integrin trafficking in different cancer cell lines (breast adenocarcinoma MDA-MB-231, prostate cancer PC-3 and non-small cell lung cancer NCI-H460). The endocytosis of integrins is also visualized sub-cellularly in different endosomal compartments using confocal microscopy. Functional perturbations of different endocytic pathways are achieved using site-directed mutagenesis of important target genes.

**Results:** Our results show both active and inactive β1α2 integrin conformers are endocytosed in a clathrin- and dynamin-dependent manner. The net endocytosis rate of the active β1 integrins is higher, whereas endocytosis of the inactive β1 integrin is counteracted by rapid recycling back to the plasma membrane via an ARF6- and early endosome antigen-1-positive compartment in a Rab11a- and actin-dependent manner. Owing to these distinct trafficking routes, the two receptor pools display divergent subcellular localization. At steady state, the inactive β1 integrin is mainly on the plasma membrane, whereas the active receptor is predominantly intracellular.

**Conclusions:** These data provide new insights into the endocytic traffic of integrins and imply the possibility of a previously unappreciated crosstalk between pathways regulating integrin activity and traffic. This helps us to further understand the complex process of adhesion receptor recycling and cell migration.

485 Lack of Caveolin-1 Mimics SHH Stimulation in Pancreatic Adenocarcinoma, Promoting EMT and Tumour Migration

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**Introduction:** Pancreatic ductal adenocarcinoma (PDA) is a heterogeneous disease with dismal prognosis. Caveolin-1 (Cav-1) and sonic hedgehog (SHH) pathway have been involved in the genesis and the progression of this disease but none has explored the possible interaction of the two. Interestingly, in vitro and in vivo studies are often conflicting on the role of Cav-1 in PDA. Here we explored the pattern of expression of Cav-1 in PDA biopsies in vivo and determined whether the levels of Cav-1 expression may influence SHH signaling in inducing EMT and cell migration in vitro.

**Material and Method:** 15 PDA biopsies were studied by IHC for Cav-1. BxPC-3 pancreatic cell line, which highly expresses Cav-1, was used in our study. Lentiviral mediated siRNA expression was employed for stable silencing of Cav-1 in the same cells. The differential expression of Cav-1 and E-cadherin was studied both with western blot and RT-PCR. Scratch assay was used to study the migration/proliferation differences between cells expressing or not expressing Cav-1. Stimulation of cells with recombinant SHH (rSHH) 2 μg/ml was performed and the effect on migration was assessed. Proliferation of cells was assessed measuring PCNA expression by western blot.

**Results and Discussion:** Pancreatic cancer biopsies showed a heterogeneous pattern of Cav-1 expression, both inter and intra patient. Interestingly the poor differentiated areas of pancreatic cancer were consistently expressing more caveolin-1 in the cancer cells and less in the stroma, whereas in moderately differentiated ones the pattern was inverted. In vitro studies showed that silencing of Cav-1 was associated with a reduction of 70% in the levels of E-cadherin both at mRNA and protein level. SHH stimulation and caveolin-1 silencing sorted a similar effect on migration by decreasing migration index at 24 hours by 147% and 155%, respectively. Nevertheless, SHH stimulation had an opposite effect on cav-1 silenced cells reducing their migration index by 56% fold. PCNA expression levels indicated no differences in cell proliferation.

**Conclusions:**

1. Modulation of caveolin-1 expression within the cancer may influence the infiltrating properties and differentiation status of the cancer cells.

2. In vitro studies on any pancreatic cell line must account for the levels of Cav-1 expression when analyzing any functional pathway.

3. Inhibition of SHH pathway may sort opposite effects in cancer expressing high or low levels of Cav-1.

4. Cav-1 IHC analysis should be included in classification and characterization of pancreatic cancer.

487 Reduced Expression of the Tumor Suppressor Spred-2 in Acute Myeloid Leukemia Patients

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**Background:** The Sprouty protein was originally identified in Drosophila melanogaster as an antagonist of Breathless, the ortholog of mammalian Fibroblast Growth Factor Receptor (FGFR). Members of this family are Sprouty and Spred-1-2 proteins which are indubitable inhibitors of signalling induced by receptor tyrosine kinases. The main function of these members is to down-regulate the RAS/RAF/ERK signalling pathway by physically interacting. Sprd, in normal subjects, is able to negatively regulate hematopoiesis but its role in haematological malignancies is still not been clarified.

**AIM:** The aim of this study was to investigate the role played by Spred-2 in acute myeloid leukaemia (AML).

**Materials and Methods:** Bone marrow (BM) cells were collected from (AML) patients at diagnosis. All cases were characterized at the cytogenetic level by conventional karyotyping, and screened by reverse transcriptase-PCR and sequencing for the presence of the most frequent fusion transcripts and mutations, respectively. We analysed Spred m-RNA by RT-qPCR. Moreover protein levels were evaluated by Western blotting and immunofluorescence. Cell proliferation and apoptosis assay were performed by overexpression of Spred-2 cDNA in Kasumi cells line.

**Results:** The analysis of mRNA Spred-2 level in 82 BM reveals a significant reductions in AML patients compared to control (mean value of expression 0.564; normal 0.186 AML p < 0.0001). There was no significant difference in Spred-2 expression among the different FAB subtypes, and in patients characterized by the presence of chromosomal translocations compared to those with normal karyotype status. This data is corroborated at the protein level; indeed the protein analysis corroborated the sharp reduction of Spred-2 in AML patients when compared to normal control, both in Western blotting and in immunofluorescence assay. Noteworthy, the achievement of complete remission detected an increasing of Spred-2 protein, comparable to those of normal subjects. Moreover, Spred-2 overexpression in Kasumi cells leads to a decreasing in proliferation (approx. 53%), colony growth formation (approx. 50%) and an increasing of the apoptotic rate (approx. 10%).

**Conclusions:** We demonstrated a decreasing of Spred-2 expression in AML patients, with the mean value of 0.564 and 0.186 in normal and AML BM, respectively. Furthermore, disease remission correlated with re-acquisition of normal levels of the protein. Therefore, we need to perform further studies of miRNA-21, one potential regulator of Spred-2, which expression is strongly elevated in a variety of human neoplastic disorders. Since Spred-2 expression appears increased in 12% of AML patients we are planning to investigate the role of such miRNA in AML patients miRNA.
correlated with P-cadherin positive lesions and CD44+/CD24−/low pattern, an associated cancer stem cell phenotype. Concerning regulatory potential of p63 isoforms, we observed their ability to differentially modulate the activity of CD43 promoter in breast cancer cells, being the truncated isoform Np63α the one which induced greater gene activity. A more complex mechanism of inhibition of CD43 promoter is suggested by usage of TA- and γ-isoforms, which showed to have dominant negative effects in the presence of Np63α. Additionally, preliminary data showed that induction of TAp63β in cells with overexpression of P-cadherin reduce significantly its expression and aggressiveness properties, such as invasion and anchorage independent survival.

Conclusions: This study contributes not only to better understand the individual role of p63 proteins in breast cancer related genes but, most importantly, it brings new insights regarding the players which regulate the invasive and stem cell properties mediated by P-cadherin in breast cancer cells.

[492] Role of Nitrogen Species in Gastrointestinal Cancer Stem Cells

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Background: Chronic inflammation represents a leading cause of neoplastic transformation in colorectal (CC) and other human cancers, in part due to DNA damage and tumor promotion by oxidant species (Reactive oxygen species, ROS) and Nitric Oxide(NO) generated at inflammatory sites. It has also been suggested that high NO synthesis, secondary to inducible NOS (iNOS or NO2) expression, are a distinctive feature of 'cancer stem cells' (CSC), a small subset of cells with self-renewal capacity, believed to be responsible for tumor initiation, maintenance and spreading. Importantly, these cells may be identified within a variety of human cancers, including CC, by the expression of the CD133 surface marker.

Aim: This study was defined to explore the contribution of NO in the definition of CSC features in colon cancer.

Materials and Methods: The production of intracellular NO will be assayed using the 4,5-diaminofluorescein diacetate (DAF-2DA) detection system. Cells were sorted from the CC cell line CaCo-2, on the basis of their CD133 expression and higher malignancy in vitro and in vivo assays compared.

Results: We have observed that CD133+ cells (putative CSC) isolated from the CaCo-2cell line, produce more NO than CD133− cells (non-CSC), and that, once sorted for intracellular NO levels, NO 'high' cells display higher CD133 expression and higher malignancy in vitro and in vivo assays compared to NO 'low' cells. Additionally, experiments with the highly selective NOS2 inhibitor 1400W and NOS2-directed shRNA, confirmed that NOS2 is critical for NO production in CaCo-2 cells.

Conclusion: These studies are relevant, since they may provide the first demonstration that NO synthesis, secondary to high NOS2 expression, is a distinctive feature of colon CSC relative to non-CSC, and in the long run, these data will hopefully serve as an important for evaluation of NOS2-directed therapies as a component of multimodal treatment regimens for human CC.

[493] SET Regulates Levels of HnRNPK and Its Binding to Nucleic Acid in HNSCC

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Background: In previous studies SET and HnRNPK proteins were found accumulated in head and neck squamous cell carcinoma (HNSCC). HnRNPK is associated with prognosis in HNSCC and other neoplasias and is considered a nucleic acid-regulated docking platform involved in RNA splicing, mRNA stability, chromatin remodeling, DNA repair and transcription. SET has roles in cell cycle regulation, chromatin remodeling, proliferation and cell death signaling. Here we addressed (i) potential interaction between SET and HnRNPK K and (ii) SET effect on HnRNPK.

Material and Method: HEK293 cells with SET overexpressed (HEK293/SET), HNSCC cell lines (HN6, HN12 and HN13) with or without SET knockdown (siSET) were used as models. Co-immunoprecipitation (Co-IP), quantitative real time PCR (qPCR) and Western blotting were used to assess SET and HnRNPK K interactions and targets. Protein-DNA interaction was analyzed by electrophoretic mobility shift assay (EMSA) using a single strand DNA oligo(dC) and protein extracts.

Results: QPCR showed hnrRNPK K mRNA (transcripts a and b) up-regulated in HEK293/SET cells and down-regulated in siSET HNSCC cells, suggesting that SET protein regulates its levels. Western blotting analysis showed that SET overexpression increased hnrRNPK K protein whereas a decrease was observed by siSET in HNSCC cells. Co-IP assays showed hnrRNPK K as a SET protein binding. Since hnrRNPK K protein binds nucleic acid and regulates Bcl-xS (pro-apoptotic) splicing, we assessed whether SET could modulate these specific hnrRNPK K functions. EMSA assays showed that SET increased hnrRNPK K-ssDNA binding. The Bcl-xS mRNA was measured by qPCR and it was decreased. SET-hnrRNPK K interaction. Diminished in Bcl-xS mRNA levels was observed in the presence of both hnrRNPK K and SET overexpressions whereas siSET in HNSCC cells reversed the effect.

Conclusion: SET regulates hnrRNPK K protein levels and also modulates its interaction with nucleic acid. Also, SET cooperates with HnRNPK K to reduce the pro-apoptotic Bcl-xS. We propose that both proteins, up-regulated in HNSCC, cooperate to tumorigenesis and disease progression.

[494] Core Ras Pathway Signaling in Human Colorectal Cancers Revealed by Isogenic Modeling of NF1, KRAS and BRAF Mutations

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Genomic analyses have implicated frequent somatic mutations in the Ras pathway genes NF1, KRAS, and BRAF in the evolution of colorectal cancers and other common malignancies such as those of the brain and lung. To identify similarities and differences in the phenotypes of common mutations in these three genes, they were engineered in the same human colorectal cancer cell lines and used to reconstitute viral knockout cell lines. This reconstituted system was then assessed to determine the target genes of interest. The activated Ras pathway was characterized by comparing resistance to low glucose growth conditions. Colorectal cancer cells having only wild type Braf functional allele has shown activated Ras pathway either by activating Kras or by Knocking out NF1 gene. These isogenic cell lines can be used to discover pathway selective drugs.

[495] Hormonal and GABA-ergic Systems in Breast Cancer Patients

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Introduction: Breast cancer is one the most common cancer in the UE. About 14000 women get breast cancer in Poland each year. -Aminobutyric acid (GABA) is the inhibitory neurotransmitter in central nervous system. It plays a role in regulating neuronal excitability throughout the nervous system. GABA is also directly responsible for the regulation of important processes. The role of GABA, GAD and GABA-ergic receptors in breast cancerogenesis and in breast cancer progression is still to be clarified.

The aim of the study was to estimate a mutual correlation between GABA-ergic system (GABA content and GAD activity) and hormonal system (prolactin and estradiol content) in breast cancerogenesis and in breast cancer progression in women. This study contributes not only to better understand the hormonal and GABA-ergic system but also to better comprehend the role of GABA in breast cancerogenesis.

Materials and Methods: The production of intracellular NO will be assayed using the 4,5-diaminofluorescein diacetate (DAF-2DA) detection system. Cells were sorted from the CC cell line CaCo-2, on the basis of their CD133 expression and their level of DAF-2DA(NO'high' and NO'low') by FACS-sorter, and thereafter drawn from the CC cell line, producing more NO than CD133− cells (non-CSC), and that, once sorted for intracellular NO levels, NO 'high' cells display higher CD133 expression and higher malignancy in vitro and in vivo assays compared to NO 'low' cells. Additionally, experiments with the highly selective NOS2 inhibitor 1400W and NOS2-directed shRNA, confirmed that NOS2 is critical for NO production in CaCo-2 cells.

Conclusion: These studies are relevant, since they may provide the first demonstration that NO synthesis, secondary to high NOS2 expression, is a distinctive feature of colon CSC relative to non-CSC, and in the long run, these data will hopefully serve as an important for evaluation of NOS2-directed therapies as a component of multimodal treatment regimens for human CC.

Results: Ductal carcinomas were diagnosed in 78% patients. T1 and T2 were observed in 85%. Grading G3 was evaluated in 51% of patients. 31% pts developed lymph nodes metastases.

The GABA content and GAD activity was higher (p<0.005) in tumor as compared to normal mammary gland tissue. There was clear positive correlation between GABA content and GAD activity in both tissue studied (p<0.05). The positive correlation between concentrations of GABA and GAD activity in T2 were mentioned (p<0.05). The measured GABA concentrations and GAD activity were higher in women who had nodal metastases (p<0.05). 94% pts developed receptors positive breast cancer. In 61% pts the presence of prolactin was observed. There was positive correlation between GABA content and GAD activity in ductal invasive breast cancertumor microscopically normal mammary gland tissue (p<0.05). No significant correlations between GABA content, GAD activity and receptor expression and hormonal status of studied women were noticed.
Tracing the Metabolic Profile of Prostate Cancer Progression
Roles for Monocarboxylate Transporters (MCTs) in Prostate Cancer Metabolism

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Background: Despite its implication in virtually all malignant cells, the role of altered cellular metabolism as an essential factor in prostate malignancy has been largely ignored. This work aims to study and identify metabolic changes associated with malignant transformation, identifying overexpression of key metabolic proteins in prostate cancer, linked to cancer progression and poor prognosis. This will allow us to infer the most important metabolic alterations in prostate cancer, giving a special focus on the role of monocarboxylate transporters (MCTs) in prostate cancer metabolism.

Material and Methods: Expression of MCTs as well as key metabolic markers involved in lipid (oxidation and glycolytic metabolism) was assessed by immunohistochrometry in 480 prostate samples and different prostate cancer cell lines. The levels of glycolytic metabolism were assessed in the cell lines using commercial colorimetric assays. The effect of the classical MCT inhibitor (cyano-4-hydroxycinnamic acid (CHC) was evaluated on cell viability by using the Sulforhodamine B assay. Cell localization of MCTs is prostate cells was assessed by co-localization with organelle markers using immunofluorescence. Finally, ultra-structural studies were performed by classical electron microscopy techniques to understand the morphology of human prostatic cancer and perhaps provide an insight into the structure-function relationship.

Results: This study revealed that prostate cancer cells overexpress a variety of key metabolic proteins and the assessment of a 'metabolic signature' appears to be indicative of prostate cancer initiation since we observed that many alterations are already evident in PIN lesions. Also, studies in cell lines support a switch from low glycolytic metabolism in the localized and less aggressive in situ tumor to high levels of glycolytic metabolism in the highly aggressive and metastatic prostate tumor. The prostate cancer cell lines showed several differences at metabolic and ultrastructural levels. LNCaP cells exhibited a more oxidative phenotype based on glucose consumption and lactate production rates, whereas PC3 and DU145 were more glycolytic. The dependence of LNCaP on oxidative phosphorylation and PC3 on glycolysis was also evident in electron microscopy studies where differences in mitochondrial content were evident. Differences in MCT expression and localization among the cell lines were also evident. The strong plasma membrane expression of MCT1 in LNCaP cells contrasted with the scattered cytosolic, almost undetectable expression of MCT1 in PC3. MCT4 was preferentially expressed in the plasma membrane of PC3 and DU145 cell lines. Differently, and for the first time we found MCT2 at the cytoplasm, associated with the peroxisome membranes. Additionally, CHC significantly decreased the viability of DU145 and PC3 cells, while LNCaP was less sensitive suggesting MCTs as possible targets for aggressive prostate cancer.

Conclusions: All together, these results suggest that a 'metabolic signature' appears to be indicative of prostate cancer initiation since we observed that many alterations are already evident in PIN lesions. Also, correlations with clinic-pathological data indicate an association between glycolytic proteins, namely MCT4 and poor prognosis. These results together might suggest a switch from oxidative metabolism in the localized and less aggressive in situ tumors to glycolytic metabolism in the highly aggressive and metastatic prostate tumor and underscores the plasticity of bioenergetic pathways in prostate cancer cells which might have crucial implications at prognostic and therapeutic level, pointing to MCTs as possible targets in prostate cancer.

Inherited mutations in genes involved in DNA repair processes, cell cycle, and apoptosis, clearly play a causal role in the development of cancer, often with an incomplete penetrance. Polygenic alleles in these genes can also contribute to the origins, propagation and treatment responses of a cancer. The TP53 codon 72 SNP (P72R) leads to a proline to arginine substitution with different capacities for inducing gene transcription, interacting with others proteins or modulating apoptosis. The insertion of 16 bp in intron 3 (PIN3) has been suggested to interfere with normal splicing and mRNA processing which leads to lower expression of P53. This study is interested in the relationship between colorectal cancer (CRC) risk and polymorphisms in TP53.

Material and Method: 98 tumors from non-related patients treated at ACCamargo Hospital/Brazil and 97 blood samples from controls were evaluated. DNA was isolated from frozen tissue tumor or blood using phenol/chloroform protocol and evaluated by sequencing. Associations were analyzed by Pearson Chi-Square or Fisher Exact tests.

Results: The minor allele frequencies for the R72P and PIN3 polymorphisms are, respectively, 0.27 and 0.13 in controls and 0.23 and 0.12 in patients, with no significant differences observed. No controls in contrast to two (2%) patients revealed de A2/A2 genotype. Both A2/A2 tumors presented TP53 mutation, p53 expression, infiltrative pattern of tumor growth, desmoplasia, infiltrating lymphocyes, are T3-T4 staging and underwent post chemoradiotherapy. Analysis of tumor samples showed that: 95.1% of Arg/Arg tumors are A1/A1; 78.9% of A1/A2 are Pro/Arg and 85.7% of A2 expressers are Proline expressers (P < 0.001). In relation of tumor behavior, 90.5% of A1 exclusive expressers didn’t presented recurrence (P = 0.035). Previous studies with this samples showed that P72R expression was significantly associated with gender, recurrence, dirty tumor necrosis, border pattern of tumor growth, post chemoradiotherapy use, p53 expression and TP53 mutation, suggesting that the presence of at least one Arg/72 allele is associated with better prognosis.

Conclusion: The results of this study provide evidence for the association of P72R and PIN3 polymorphisms. There also appears to be a specific association between the risk of CRC in patients harbouring polymorphisms in TP53. Consideration of LNCaP in tumor heterogeneity is therefore important in the critical analysis of gene associations in cancer.

Sulindac Compounds Facilitate the Cytotoxicity of β-lapachone by Up-regulation of NAD(P)H Quinone Oxidareductase
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Introduction: Lung cancer is common and the most cause of cancer death in Taiwan. β-Lapachone, a novel bio-oxidoreductive drug, exhibits cytotoxicity to cancer cells overexpressing NAD(P)H: quinone oxidoreductase-1 (NQO1). NQO1 is an important enzyme that reduces quinones to hydroquinones for preventing the generation of the semiquinone radical. Sulindac and its metabolites, sulindac sulfide and sulindac sulfone, are non-steroidal anti-inflammatory drugs and be suggested to increase the expression and enzyme activity of NQO1. In this study, we investigated the synergistic cytotoxicity of combined treatment of sulindac compounds and β-lapachone to human lung cancer cells.

Material and Method: We examined the role of NQO1 in β-lapachone-mediated cytotoxicity to lung cancer cell lines and evaluated the anti-cancer efficacy of combined treatment with sulindac compounds and β-lapachone using MTT assay, RT-PCR, western blots, and DNA fragmentation methods.

Results and Discussion: The levels of NQO1 expression and activity in lung cancer cell lines are A549 > CL1-1 > CL1-5. Exposure of CL1-1 and CL1-5 cells to β-lapachone resulted in survival inhibition and apoptosis. This increase in apoptosis was associated with a decrease of mitochondrial membrane potential suggesting the loss of mitochondrial function by β-lapachone. In addition, the induction of calcium influx in β-lapachone-treated cells triggered the ER stress and the inhibition of PI3K/Akt signaling pathway. However, all of these events was dependent upon NAD(P)H: quinone oxidoreductase (NQO1) activity. The treatment of sulindac compounds including sulindac, sulindac sulfide and sulindac sulfone significantly upregulated the NQO1 expression and NQO1 activity in all lung cancer cell lines we tested. Furthermore, the combination of sulindac or sulindac derivates and β-lapachone has synergistic cytotoxic effects on lung cancer cells. Inhibition of NQO1 activity with NQO1 inhibitor, dicumarol or decreasing NQO1 mRNA and protein levels with NQO1 siRNA significantly reduced the NQO1 expression and thus attenuated the cell death by combined treatment of sulindac compounds and β-lapachone.

Conclusion: We suggested that sulindac compounds significantly increase the endogenous NQO1 expression and enzyme activity and thus enhance the β-lapachone-mediated lung cancer cell death.
Apoptosis Repressor With a CARD Domain (ARC) is Essential for the Survival of VHL Deficient Renal Cancer Cells

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Background: The induction of hypoxia inducible factors (HIFα) is essential for the adaptation of normal and tumor cells to a low oxygen environment. In contrast, clear cell renal carcinoma (CC-RCCs) are characterized by loss of the Von Hippel Lindau (VHL) tumor suppressor gene, allowing for new therapeutic avenues based on targeting the corresponding proteins and triggering apoptotic death of cancer cells.

Materials and Methods: Cells were subjected to hypoxia (0.5% or 2% O2) and anoxia (0.02% O2) in Hypoxic/Anerobic chambers. mRNA and protein expression were assessed. Furthermore, ARC was a transcripive Repressor of androgen receptor (AR) and Western blotting respectively. Small interfering RNA (siRNA) transfections were performed using specific siRNA smart pools, and Dharmafect reagent 1 from Dharmacon. PKM2 knockdown expression constructs from Open Biosystems, along with packaging plasmids pR8.2 and pVSVG, were used for lentivirus production in 293FT cells. For immunohistochemistry kidney tissue samples were obtained from the antigen retrieval in sodium citrate based buffer, followed by biotin-streptavidin-HRP amplification and visualization. For in vivo experiments SCID mice were injected subcutaneously into the dorsal flanks with Caki1 cells stably expressing siRNA constructs. Tumor volume was calculated at 13 weeks after cell implantation (width3×length ×0.5).

Results: We found that an apoptosis inhibitor ARC was induced by hypoxia in a variety of cancer cell types and was highly expressed in normoxic VHL-deficient CC-RCCs in a HIFα-dependent manner. The increased ARC expression in human kidney cancer tumor samples compared to normal kidney tissue supported the importance of ARC deregulation in human kidney cancer. To understand the role of ARC in kidney carcinogenesis, we evaluated the effects of ARC inhibition in human CC-RCC cell lines and in vivo. Genetic inhibition of ARC expression by RNA interference in CC-RCCs led to decreased colony formation ability and increased apoptosis, as well as a dramatic reduction of tumour growth in SCID mice.

Conclusion: Our findings establish ARC as a novel pro-tumorigenic effector of HIFα signaling, and suggest that targeting ARC is a promising therapeutic direction in human kidney cancer.

Expression Pattern of TSPY and TSPX Proteins in Cases of Testicular Tumours

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Introduction: The TSPY is the evolutionary conserved multi-copy gene family linked to Y chromosome with function in early spermatogenesis, immediately prior to the spermatogetonia-to-spermatocyte transition, and in early testicular tumorigenesis. Expression of TSPY has been reported in various solid tumours, including germ cell tumours (gonadoblastoma, seminoma, carcinoma in situ, etc.) and somatic tumours (melanoma, hepatocellular carcinoma, epithelial prostate cancer, etc.). TSPY protein is known to positively regulate cell cycle progression, in particular regulating G2/M transition, by interaction with cyclin B. Within this interaction TSPY competes with its X chromosome homologue – TSPX. On the contrary, the TSPY exerts the function of cell cycle inhibitor by transcriptional up-regulation of p21 associated with ERK1/2 kinase. These activities are controlled by PKM2, an isoform of TKT, an enzyme participating in the glycolytic pathway.

Materials and Methods: Indirect immunohistochemistry staining of TSPY/TSPX on formalin fixed paraffin embedded testicular tumour samples (92) were assessed and divided based on the diagnosis given: (i) 50 seminoma samples; (ii) 22 embryonal carcinoma samples and (iii) 20 mixed germ cell tumour samples

Results and Discussion: Our data showed enhanced nuclear positivity for TSPY and TSPX in basal parts of normal seminiferous ducts, as well as in intratubular germ cell neoplasia (IGCN). We found statistically significant difference in both, nuclear and cytosolic positivity of TSPY in seminoma (high TSPY) and embryonal carcinoma (low TSPY). Loss of nuclear TSPY expression was in correlation with decreased TSPX nuclear expression in seminoma.

Conclusion: Our study supports the role of the nuclear TSPY and TSPX expression as a diagnostic markers for differential diagnostic of seminoma and embryonal carcinoma samples. Our results were supported by grant GA410/03/08/H048, IGA UP LF_2012_019 and Operational Programme Research and Development for Innovations (project CZ.1.05/2.1.00/01.0030).

Insulin Upregulates PKM2 Expression But Inhibits its Activity to Promote Cancer Cell Metabolism

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Background: Hyperinsulinemia has been linked with high incidence of liver and other cancers. However, the mechanism into insulin’s role remains less clear. Recent studies have shown key role of pyruvate kinase M2 (PKM2) in cancer metabolism and growth.

Materials and Methods: Insulin sensitive HepG2 cells were used for experiments. PKM2 expression was analyzed using real time PCR and Western blotting. Glycogenic status of PKM2 was assessed using gradient centrifugation. Metabolic behavior of cells was studied using commercially available kits. ROS was analyzed using fluorescence spectrophotometry.

Results: Here, we show that insulin upregulated PKM2 expression but decreased its activity in HepG2 cells. Decrease in PKM2 activity was attributed to shift in critical dimer-tetramer equilibrium towards lower activity dimeric PKM2, assessed by density gradient centrifugation. Interestingly, insulin induced increased PKM2 activity in PKM2 activity independent. Notably, phosphorylation of PKM2 at tyrosine 105 remained unaffected on insulin treatment. Further, decreased PKM2 activity resulted in characteristic pooling of glycolytic intermediates and increased NADPH synthesis; suggesting diversion of glucose flux towards de novo glucose synthesis, required for cancer cell growth. Aerobic glycolysis was increased upon insulin treatment. PKM2 knockdown confirmed that insulin promoted aerobic glycolysis through PKM2. Further, we provide an evidence for the involvement of insulin induced ROS in inhibition of PKM2 activity.

Conclusion: Collectively, this study identifies new PKM2-mediated effects of insulin and thus advances the understanding of insulin’s role in cancer.

Resveratrol Down Regulates Transketolase Like-1 to Inhibit Cancer Metabolism and Cellular Growth

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Background: Metabolic requirements of cancer cells essentially differ from their normal counterparts. Cancer cells produce increased lactate even in presence of oxygen. This metabolic transformation is referred to as aerobic glycolysis; hallmark of cancer metabolism. Since the metabolism, in cancer cells, is diverted to anabolism in via up regulation of biosynthetic pathways, expression of enzymes involved in these pathways becomes a critical factor to drive the metabolic fate. The pentose phosphate pathway (PPP) is the predominant pathway for converting glucose to ribose sugar for nucleotide synthesis. Transketolase (TKT) enzyme regulates the non oxidative branch of PPP and thus is known to play a contributing role in PPP. Transketolase like 1 (TKTL1), an isoform of TKT, is known to play an important role in cancer metabolism.

Materials and Methods: mRNA and protein levels were analyzed by qPCR and western blotting respectively. Glycose uptake and lactate production were assessed using kits according to manufacturer’s protocol. Knockdown experiments were done using TKTL1-shRNA.

Results: We observed substantial decrease in expression of this enzyme both at mRNA and protein level. We assessed the implications of down regulation of TKTL1 on glucose uptake and lactate production (aerobic glycolysis) in experimental cancer cell lines. We observed a significant decrease in aerobic glycolysis. Since Resveratrol is known to inhibit glucose uptake and lactate production, we transiently silenced TKTL1 and then assessed the glucose uptake and lactate production to show that TKTL1 at least in part, affects aerobic glycolysis. Interestingly, aerobic glycolysis was negatively affected upon TKTL1 silencing. Furthermore, cellular growth was also retarded. Results strongly suggested that the negative effects of Resveratrol on aerobic glycolysis are partly mediated by TKTL1.

Conclusion: Collectively, our data suggest that nuclear TKTL1 expression negatively affects expression of Resveratrol and further endorses it as a promising anti-cancer drug.
A Comparative Study of Various FLT3-ITD Mutations

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Internal tandem duplications (ITD) in the tyrosine kinase receptor FLT3 (FMS Like Tyrosine Kinase) is one of the most common class of mutations in Acute Myeloid Leukemia (AML) and presence of mutations indicates a poor prognosis. However, FLT3-ITD mutations found in patients may vary from 3 up to hundreds of nucleotides and may be located in the juxta-membrane domain and the tyrosine kinase 1 domain. Considering the substantial differences in lengths of various FLT3-ITDs detected in AML patients we have performed a comparative study of various FLT3-ITD mutations isolated from AML-patients regarding transformation and proliferation capacity, growth and survival signalling and response towards FLT3 targeting inhibitors. Hematopoietic progenitor cell lines or murine primary cells were used in these experiments.

Sunday 8 – Tuesday 10 July 2012
Poster Session
Cancer Genomics, Epigenetics and Genomic Instability

Identification of Fusion Transcripts in Colorectal Cancer by Combined RNA-seq and Exon Microarray Analyses
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Background: Colorectal cancer (CRC) has a world incidence of more than one million, and almost half of the patients die from their disease. There is thus an obvious lack of CRC specific drug targets and clinically useful biomarkers. Cancer specific fusion transcripts and subsequent fusion proteins represent promising molecules in this regard. However, only one recurrent CRC fusion transcript has been described, namely TCP7L2-VTT1A, in 3% of patients.

Materials and Methods: In the current study, we searched for recurrent fusion transcripts in CRC by paired-end RNA sequencing combined with analyses of exon microarray data. We used the RNA-seq approach to analyse seven CRC cell lines, and searched for fusion transcripts by the deFuse software. To search for recurrent fusion transcripts, we tested for differential intra-genic expression, up- and downstream of each indicated fusion breakpoint, by analysis of exon microarray data from 221 CRCs.

Results: A list of 3391 resulting fusions were filtered by removal of chimeric transcripts present in RNA-seq data from normal tissues and genes with multiple partners within the same samples. This nominated 212 fusion transcripts from the CRC cell lines. Additional filtering of fusions with partners at least 100 kbp apart and where both partners had breakpoints exactly at exon-intron boundaries resulted in 11 fusion genes, of which 10 have been experimentally verified by RT-PCR and Sanger sequencing. Analysis of exon-intron boundaries resulted in 11 fusion genes, of which 10 have been experimentally verified by RT-PCR and Sanger sequencing.

Conclusion: We have developed an analysis pipeline for identification of cancer specific fusion transcript biomarkers, where the identification stage is performed by RNA-seq, and the validation stage with search for recurrent events is performed by analysis of larger number of samples analysed by exon-resolution microarrays.

The GATA2 Transcriptional Network is Requisite for Ras Oncogene-driven Non-small Cell Lung Cancer
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Introduction: We have performed an RNA interference screen to discover factors required for mutant cancer cells; among the factors discovered was the transcription factor GATA2.

Material and Method: To systematically define the role of GATA2 in Ras-driven NSCLC, we performed an integrative analysis of GATA2 in Ras-pathway mutant and wild type NSCLC cells via (1) gene expression with and without GATA2 depletion by shRNA; (2) GATA2 genome occupancy via next-generation sequencing; and (3) modulation of GATA2-regulated pathways in the presence or absence of GATA2 loss. To assess the function of GATA2 in autophichous lung tumourigenesis, we combined genetically engineered mouse models (GEMMs) of Kras-driven NSCLC with a conditional allele of Gata2. Finally, to explore whether drugs inhibiting GATA2-controlled pathways suppress lung tumourigenesis, we treated animals from the Kras-mutant GEMM with clinically licensed inhibitors of the proteasome and Rho kinase.

Results and Discussion: An examination of a panel of 26 NSCLC cells revealed GATA2 loss induced a specific lethality in KRAS cancer cells. Intriguingly, NSCLC cells with additional mutations in the Ras pathway also exhibited sensitivity to GATA2 depletion. Through integrated genomic analysis of gene expression and genome occupancy, we discovered GATA2 transcriptionally controlled multiple signalling pathways, including the proteasome and Rho signalling cascades. Functional examination revealed each pathway to contribute to GATA2-mediated viability, while none was necessary for Ras-driven NSCLC survival. We then assessed the role of Gata2 in Ras-driven cancer in vivo with GEMMs of Kras-driven NSCLC. Intriguingly, loss of Gata2 robustly suppressed both the initiation and maintenance of these lesions. We then analysed the effect of inhibiting GATA2-regulated pathways by treated the Kras-mutant GEMM with clinically licensed inhibitors of the proteasome (bortezomib) and Rho kinase (fasudil). Combined inhibition of these pathways led to a striking reduction in tumour burden in vivo.

Conclusion: Our findings define GATA2 as the regulator of a transcriptional network required for the survival of Ras-driven NSCLC [1]. Importantly, while transcription factors like GATA2 may not appear to be druggable targets, integrative analysis has revealed a new paradigm of targeting orthogonal pathways in cancer.

Reference(s)

Aberrant Androgen Response Underlies Altered Expression and Epigenetic Regulation of Endogenous Retroviruses in Human Prostate Cancer
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Background: Since reactivation of human endogenous retroviruses (HERV) is thought to contribute to cancer progression, we determined expression, epigenetic state and androgen dependency of specific HERV-K proviruses in prostate cancer.

Materials and Methods: HERV-K expression was analyzed in human prostate cancer cell lines and a well defined set of 11 benign and 45 cancerous prostate tissues by qRT-PCR. DNA methylation was quantified by pyrosequencing. Histone modifications were assessed by chromatin immunoprecipitation (ChIP). Androgen responsiveness was determined in cell lines treated with bicalutamide or methylxanthine by ChIP against androgen receptor (AR) and by reporter gene assays.

Results: Expression of the HERV-K, 22 q11.23 provirus was strongly and broadly increased in cancer tissues (p<0.001), correlating highly significantly with the expression of the ERG oncogene (p<0.001). In parallel, DNA methylation in its long terminal repeat (LTR) decreased (p<0.001). Conversely, HERV-K7 expression was significantly diminished in cancer tissues (p=0.0025), but this decrease was unrelated to LTR methylation or ERG mRNA levels. Expression of both proviruses was restricted to androgen-responsive prostate cancer cell lines. Their LTRs were found to contain steroid hormone responsive elements, bind the AR in vivo and confer androgen responsiveness to reporter constructs.

Conclusions: Our findings indicate a surprisingly high specificity of changes in expression and DNA methylation of androgen-dependent HERV-K proviruses in prostate cancer, which appear to be related to the aberrant androgen response in cancers with ERG rearrangements. Supported by the Deutsche Forschungsgemeinschaft.

Epigenetic Mechanisms Involved in Concomitant Downregulation of the Oppositely Regulated Genes DLK1 and MEG3 at 14q32.2 in Urothelial Carcinoma
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Background: In invasive urothelial carcinoma (UC), the chromosomal region 14q32.2 is often deleted. Two imprinted genes, Delta-like 1 (DLK1) and MEG3 are oppositely regulated via two differentially methylated regions (DMR). DLK1 and MEG3 expression and regulation have not been studied in UC.

Materials and Methods: DLK1 and MEG3 expression was investigated by qRT-PCR in cancerous and benign bladder tissues, UC cell lines and normal urothelial cells. Gene copy numbers were analysed by qPCR. DNA methylation by bisulfite sequencing and pyrosequencing. Histone modifications were

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Genetic Aberrations in CDKN2a and TP53 Genes in Patients with Laryngeal Carcinoma
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Background: Laryngeal squamous cell carcinoma (LSCC) is the second most common tumour of the head and neck. It is characterized by high rate of developing local or regional recurrence and second primary tumours. Thus, it is of critical importance to understand the molecular basis of laryngeal tumorigenesis. A common feature of laryngeal carcinomas is the inactivation, mainly by mutations, of two tumour-suppressor genes that are involved in cell cycle regulation - CDKN2A and TP53.

Methods: In the current study we aimed to examine mutation status of CDKN2A and TP53 genes in a group of 60 patients with laryngeal carcinoma. DNA was extracted from fresh-frozen tumour tissues and exons 1 to 3 of CDKN2A and exons 5 to 8 of TP53 were screened for mutations by direct sequencing.

Results: Genetic aberrations in CDKN2A were found in 7 patients (11.7%) and those in TP53 were present in 30 tumours (50%). All but one of the mutations detected in CDKN2A were not described previously. These included two insertions in exon 1 (positions 34 and 58) and one deletion of 8 bp at position 238. One missense (c.279C>G) and two nonsense mutations (c.216C>T, c.216C>A) were present in 2 exons. Two of these mutations were detected in tumours of T stage I and II, implying that they occurred during the cancer progression.

Conclusion: Screening for genetic aberrations of patients with carcinoma of the larynx showed a number of novel mutations in CDKN2A and TP53 genes. Our results could contribute to the knowledge about laryngeal carcinogenesis.

A Fra-1-regulated Gene Expression Network Required for Maintenance of Epithelial-mesenchymal Transition in Human Colon Cancer Cells
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Background: Colorectal cancer (CRC) is the third most common cancer worldwide with invasion and metastatic spread as primary causes of death from this disease. 

Fos related antigen-1 (Fra-1) is a component of Activator Protein-1 (AP-1) transcription factor complexes that is frequently over-expressed in a variety of cancers, and our group and others have shown that high Fra-1 levels play a critical role in driving CRC cell migration and invasion. 

The aim of this study was to unravel genetic programs orchestrated by Fra-1 and identify components regulated by this transcription factor in invasive CRC cells.

Material and Methods: All studies were performed with highly invasive KRAS/BRAF mutant BE CRC cells. Affymetrix Gene 1.0 ST arrays were used for gene expression profiling and chromatin immunoprecipitation (ChIP) followed by high throughput sequencing using Illumina Genome Analyzer II to identify genomic loci occupied by Fra-1. 

Results: Stable silencing of Fra-1 expression in BE CRC cells resulted in dramatic morphological changes, with the cells switching from a mesenchymal phenotype to an epithelial-like appearance and complete inhibition of cell migration and invasion.

To determine how Fra-1 silencing invokes this striking phenotypic switch, we searched for its transcriptional targets in BE cells using a combination of expression arrays to identify Fra-1-regulated genes, and ChIP-Seq (ChIP assay coupled with high throughput sequencing) to identify genomic binding sites of Fra-1. We found that Fra-1 is bound in close proximity to the transcription start site of many genes identified in the expression arrays and that the largest ontological class of genes overrepresented in both datasets encoded proteins involved in a process describes as epithelial-mesenchymal transition (EMT) (herein termed Fra-1EMT genes). Amongst these, genes associated with a mesenchymal expression profile were suppressed upon Fra-1 silencing, whereas genes fitting an epithelial profile were up-regulated. Thus, stable silencing of Fra-1 leads to a reversal of EMT in BE cells.

To determine whether changes in the expression of Fra-1EMT genes occur in primary CRCs, we performed unsupervised clustering on existing microarray data from 185 stage B and C CRCs. This analysis demonstrated that the Fra-1EMT signature is associated with poor prognosis in CRC patients. Interestingly, histochemical staining of Fra-1 in CRC specimens revealed that Fra-1 expression is significantly enriched in budding tumour cells. Tumour buds represent the invasive front of colorectal tumours and more importantly are thought to be the histomorphological hallmarks of EMT in CRCs.

Conclusions: In this study we demonstrate that Fra-1 is a key regulator of transcriptional programs required to maintain EMT in CRC cells. Furthermore, this is the first report that shows a direct link between the pattern of Fra-1 expression and EMT at the invasive front of CRC, and thus establishes Fra-1 as a new histochemical marker for a subset of highly aggressive CRCs.

Alterations of Genome Methylation Impact Tumoral Progression in Human Prolactinoma
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Alterations of DNA methylation pattern, and especially methylation of promoter, are described as one of the key mechanism involved in tumoral initiation. Nevertheless, the role of these changes in tumoral progression remains unclear. To provide new clues about the role of DNA methylation alterations in tumoral progression, we carried out the impact of promoter methylation on gene expression in non-aggressive (NA) and aggressive including malignant (A) human prolactinomas.

To investigate this fundamental question, we used a multi-dimensional integrative strategy combining transcriptomic and epigenetics data obtained on the same human tumors using genome wide approaches. Global DNA methylation profiling was realized using whole genome bisulfite-sequencing by NGS technology (HiSeq 2000, Illumina®) on one A and one NA tumor. Transcriptomic activity was assessed using Codeink® Human Whole Genome microarray on the same tumors and was confirmed on an extensive cohort. 

Methylation levels of distal promoters (10kb to 1 kb from TSS) and TSS regions (1kb to +1kb from TSS) were compared between the two phenotypes of tumors. It revealed that 1016 distal promoters and 800 TSS regions were differentially methylated between A and NA tumors. Moreover, the majority of the differentially methylated regions were hypermethylated in A vs. NA tumor. Integrative analysis between transcriptomic activity and methylation patterns showed that about 20% of differentially methylated TSS regions and 16% of differentially methylated distal promoters were inversely correlated to gene expression variation. Interestingly this analysis also highlighted that hypermethylation of TSS region but not distal promoter was linked to enrichment in down-regulated genes. Expression of genes regulated by differential DNA methylation between the two tumors was investigated on an extensive cohort leading to the selection of genes with similar expression levels. 

Here we performed the first integrative analysis including whole genome bisulfite sequencing and transcriptomic activity on the same human PRL tumors. Interestingly, our results suggested that methylation information at the TSS region is more efficient for gene repression than distal promoter methylation. Moreover, this study allowed the fine selection of specific genes on the basis of their promoters’ methylated pattern. It appeared that these genes are mainly involved in proliferation control and linked to malignant progression and would be precisely analyzed on more prolactinomas to confirm the involvement of DNA methylation in their deregulation.
Genomic Instability is a Hallmark Feature of Serous Epithelial Ovarian Cancer and May Contribute to MicroRNA Dysregulation

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Introduction: Genomic instability is a hallmark of ovarian cancer. MicroRNAs (miRNAs) are small RNA molecules that negatively regulate gene expression and are often dysregulated in cancer. The aim of this study is to determine whether copy number variations (CNV) lead to altered miRNA expression and downstream effects on target gene expression in serous epithelial ovarian cancer (SEOC).

Materials and Methods: CN, mRNA and miRNA expression profiling was performed on 4 SEOC (OV167, OV202, OVCAR-3, PE01) and 1 normal (HEK293) sample using Affymetrix Human Exon 1.0 ST arrays and Exiqon MIRCURY® U13 LNA arrays respectively. Chromosomal positioning of miRNAs was performed using Bowtie v 0.12.7 [1].

Results and Discussion: We determined that 60% (361/605) of miRNAs assessed were in regions of CNV in at least 2 cancer cell lines. Of these miRNAs, 32% (115/361) has changes in expression levels that correlated with CNV. One of these, miR-23a, is predicted to target AXL, an oncogenic receptor tyrosine kinase. The AXL/KIT signaling pathway is over-expressed in ovarian cancer and an inverse relationship was identified between the expression of AXL and miR-23a in 2 of the 4 cell lines. AXL has been previously reported to be over-expressed in 73% of ovarian tumor samples [2]. Transient transfection of miRNA mimics (Ambion) into ovarian cancer cell lines confirmed AXL down regulation after miR-23a addition. Conclusion: We demonstrate that approximately one third of miRNAs that are located in regions of CNV have altered expression which correlates with the chromosomal change. We conclude that genomic instability may contribute to miRNA dysregulation in ovarian cancer.

Reference(s)

Genetic and Epigenetic Alterations of PTPRD in Hepatocellular Carcinoma

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Background: Hepatocellular carcinoma (HCC) is one of the most deadly malignancies worldwide. Therefore detailed knowledge of the genetic and epigenetic aberrations in hepatocarcinogenic process is vital. The use of DNA microarrays has increased ability to detect deletion and amplifications and possible genes located in these regions that might be involved in HCC development. Our SNP microarray analysis of 14 HCC cell lines revealed some chromosomal aberrations. One of the most remarkable aberrations was a homozygous deletion located at prob 23 harboring PTPRD gene. PTPRD (Protein Tyrosine Phosphatase, Receptor Type, D) is frequently inactivated through deletions, mutations or epigenetic mechanisms and already regarded as a tumor suppressor gene. In this study, we investigated genetic and epigenetic alterations of PTPRD gene in human HCCs.

Material and Methods: A panel of 14 HCC cell lines were systematically screened for genome-wide chromosomal aberrations using 10K SNP microarray analysis. Mutation analysis of PTPRD gene were performed by direct sequencing in HCC cell lines and archival HCC samples. PTPRD expression was investigated in HCC cell lines and in commercially available HCC samples by using multiplex semiquantitative RT-PCR, quantitative RT-PCR and immunohistochemistry. Our SNP microarray analysis (COBRA) was used to analyse PTPRD promoter methylation status. PTPRD mRNA expression was rescued with DNA methyltransferase inhibitor (5-AzaC) and/or histone deacetylase inhibitor (TSA) treatments.

Results: Our SNP microarray study revealed a homozygous deletion site (~1Mb) at 5′ UTR region of PTPRD in one HCC cell line (Mahavu). Also, according to our genomic PCR analysis, eight coding exons of PTPRD were deleted only in Snu475 cell line which is in concordance with Sanger SNP array data. We analysed three exons of PTPRD for mutations; exon 19, 20 and 28, which have been shown to be commonly mutated in various cancers. We found only one SNP (rs10977171, Q447E) at exon 20 in one liver sample and in Snu152 cell line. We found that PTPRD mRNA expression was very low or absent in six out of 14 HCC cell lines and significantly reduced in 19 out of 23 (62.6%) primary HCCs to normal liver tissues (P-value = 0.013). According to our preliminary immunohistochemical results, level of PTPRD protein expression was also lower in tumor part of the HCC tissue compare to adjacent normal tissue. Our COBRA assay results showed tumor specific promoter hypermethylation in 27% (22%) normal-tumor paired HCC samples. We restored the PTPRD mRNA expression in Hep3B and PLC cell lines by 5-AzaC and TSA treatments.

Conclusions: PTPRD is partially deleted and epigenetically downregulated in human HCCs and suggested to be involved in hepatocarcinogenesis.

MLPA Analysis of 1p/19q Deletion in Patients With Astrocytoma and Oligodendrogliomas

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Background: A genetic hallmark of oligodendrogial tumours is the combined chromosomal deletion of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q). Combined losses of 1p/19q are also found in approximately 50% of oligoastrocytomas. These aberrations are associated with favourable prognosis, improved overall survival and chemosensitivity not only in oligodendrogliomas, but also in astrocytomas even though they are rarer in astrocytic tumours. Mutations in the IDH1 and IDH2 genes (coding for isocitrate dehydrogenase enzymes) in astrocytic tumours are commonly found together with loss of 1p and 19q.

Methods: In this study, 12 glial tumours (6 astrocytomas with IDH1 mutation and 6 oligodendrogliomas) were analyzed for 1p/19q loss by MLPA technique. MLPA analysis of large genomic deletions within chromosome 1p and 19q was performed using SALSA MLPA kit P088 which is specific for these regions. The data were interpreted using an Excel-based program which facilitates calculation of the results.

Results: MLPA analysis showed loss of 1p and/or 19q in 9 out of selected 12 glial tumours (75%). Only 3 gliomas (2 astrocytomas and 1 oligodendroglioma) showed no chromosomal aberrations in 1p/19q region. Codeletion of 1p and 19q was found in 6 patients (50%). The most frequent chromosomal deletion observed in our study was the partial loss of 1p chromosomal arm – 7 tumours. We also found chromosomal loss of 1q in one patient with glioblastoma. Because of the small groups of patients, no statistically significant conclusions about survival benefit could be made.

Conclusion: Chromosomal deletions of 1p and 19q are likely to be a frequent aberration in Bulgarian patients with oligodendrogliomas and astrocytomas with 1p/19q mutation. Further studies are required to evaluate the clinical importance of 1p/19q loss.

Promoter Hypermethylation of HIST1H4K and RASSF2 in Urine From Prostate Cancer Patients

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Introduction: Prostate cancer (PC) is the most commonly diagnosed cancer and is the second leading cause of cancer death in men. Current diagnosis of PC relies on PSA screening and/or digital rectal examination. Due to low specificity of the PSA testing there are high number of unnecessary biopsies and many undiagnosed cases. PC is a polygenic disease accompanied by many epigenetic changes. Hypermethylated DNA can be detected in body fluids from PC patients and thus it may serve as useful noninvasive biomarker for cancer diagnosis.

Materials and Methods: Using MethyLight technology we analyzed promoter hypermethylation of RASSF2 and HIST1H4K genes in urine from 57 patients with PCA and 72 controls, 43 of which were young asymptomatic men and 29 were patients with benign prostatic hyperplasia (BPH). Our goal was to determine the role of these genes as new non invasive diagnostic and prognostic biomarkers in Bulgarian PC patients.

Results and Discussion: RASSF2 did not show any methylation either in the patients or in the two control groups, which is in contrast with the findings of previous studies. This result might be due to differences in the investigated populations or the used methods.
Our results demonstrated that the methylation of HIST1H4K most likely increases with the age. We do not observe any methylation among the young asymptomatic males, while the frequency of HIST1H4K methylation did not differ between the PC patients and BPH controls. The promoter hypermethylation of HIST1H4K shows weak correlation with high Gleason score, but in order to clarify the role for the development of aggressive PC, further investigation in enlarged sample of patients and BPH controls is needed.

In our study HIST1H4K did not show correlation with PSA and the tumour stage. The contribution of this gene for development of metastases needs further investigation.

Conclusions: The promoter hypermethylation of HIST1H4K is most likely associated with the appeared with age changes in the prostatic gland and shows weak correlation with high Gleason score. Further investigations are needed in anaplastic bridge formation and spindle assembly checkpoint gene expression.

Material and Methods: OE33P and the passage matched control OE33P cell lines were cultured with ACM from obese and nonobese patients. We assessed anaphase bridges in both lines and quantified the number of bridges between the PC patients and BPH controls. The promoter hypermethylation events: anaphase bridge formation and spindle assembly checkpoint gene expression.

Materials and Methods: OE33P and the passage matched control OE33P cell lines were cultured with ACM from obese and nonobese patients. We assessed anaphase bridges in both lines and quantified the number of bridges present over the total cell number. Expression of five SAC genes (MAD2L2, BUB1B, CDC20, CENPE, and ESPL1) was assessed using qPCR. Clonogenic survival following radiation was determined in both cells lines following ACM treatment.

Results and Discussion: OE33P and OE33P showed a significant increase in anaphase bridges in response to ACM (p < 0.05, < 0.001 respectively). This increase in anaphase bridge formation was three times greater in the resistant line (p < 0.05). Levels of anaphase bridges correlated with obesity status (BMI R = 0.811 p = 0.01, Waist Circumference R = 0.706 p = 0.03, VFA R = 0.797, p < 0.006). OE33R cells treated with ACM showed significantly increased expression of the SAC genes MAD2L2 and BUB1B compared to controls (p < 0.01). Expression of MAD2L2 and BUB1B correlated with obesity status (MAD2L2: Waist Circumference R = 0.649 p = 0.05, BUB1B: BMI R = 0.733 p = 0.04, Waist Circumference R = 0.716, p < 0.03). OE33P cells treated with ACM showed increased radioreistance (p < 0.05). In contrast, in the resistant OE33R line, ACM treatment reversed this radioreistance (p < 0.001).

Conclusion: Obesity drives genomic instability and alterations in SAC gene expression in resistant SAC and alters radioreistance in SAC.

Table 1. A summary of methylation percentage and IDH1 mutation.

<table>
<thead>
<tr>
<th>MGMT gene promoter methylation</th>
<th>IDH1 mutant</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>% methylation</td>
<td>% IDH1 mutant</td>
<td>n</td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>44</td>
<td>56.7%</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>14</td>
<td>63.6%</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>11</td>
<td>57.9%</td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma</td>
<td>3</td>
<td>42.9%</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Placitc astrocytoma</td>
<td>9</td>
<td>100%</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>0</td>
<td>0.0%</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
<td>63.1%</td>
</tr>
</tbody>
</table>

Figure 1: A graphical representation of the MGMT promoter methylation levels in different tumor types.

Statistical analysis: Multivariate linear regression was used to identify associations between methylation patterns and coded smoking status, adjusting for age and batch. P-values less than p < 1 x 10^-5 were considered to be significant at the level of epigenetic-wide significance.

The same statistical method was used for the pyrosequencing data and p-values less than 0.05 were considered to be significant.

Results and Discussion: For the EWAS analysis, we included both cases and their matched controls (n = 362). We identified 17 and 15 loci in the Breast Cancer and Colon Cancer EWAS, respectively, which were differentially methylated between smokers, former smokers and those who had never smoked; eight of these loci were shared by both studies. Bisulfite pyrosequencing in a further independent population (n = 180) confirmed the results for five CpG sites identified by the 450K array (2 CpG sites in AHR, 2 CpG sites in INH and 1 CpG site in F2RL3).

Conclusions: The protein encoded by AHRH participates in the aryl hydrocarbon receptor (AHR) signaling cascade, which mediates dioxin toxicity, and is involved in regulation of cell growth and differentiation. It functions as a feedback modulator by repressing AHR-dependent gene expression.
negative feedback loop due to the induction of AHR pathway by dioxin-like compounds. Our results regarding a key gene in the AHR pathway will have relevance beyond photocytometry exposure as the key role played by this pathway in the metabolism of many environmental carcinogens.

526 DNA-index Distributions by Flow Cytometry in Canine Cancers - Indicate Variations in Genomic Instability
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Background: Pathogenesis and clinicopathological features of spontaneous cancers in the dog are very similar to their counterparts in the human, while the course of the disease is much more rapid, enabling quicker collection of prognostic data. Gross (>5%) deviations of the nuclear DNA content measured by flow cytometry (FCM) may be related to prognosis and may provide clues into the impact of tobacco smoke exposure, relevant to tumorigenesis itself.

Materials and Methods: FCM analysis of the DNA index (DI) was performed in frozen samples from canine benign and malignant cancers obtained at surgery or after euthanasia.

DNA ploidy incidence and DI distributions of mammary and thyroid carcinomas, malignant lymphomas and osteosarcomas plus soft tissue sarcomas, from dogs affected with spontaneous tumors, were assembled and compared to published data of human cancers in the human.

Results: In canine mammary as well as thyroid carcinomas the DNA-aneuploidy incidence was about 60%, which resembles the situation in the human, while more (18%) non-malignant mammary tumors were aneuploid. In close to 20% of cancers their was a significant difference in the DNA-index between primary tumor and metastases collected in the same animal. Yet, the DI-distribution in these carcinomas in the dog, was at variance with that in the human, with more frequent hypoploidy and lower level hyperploidy.

In sarcomas, some types, such as osteosarcoma, were more often aneuploid than other types such as fibrosarcoma, with large variation amongst STS. Again, hypoploidy occurred more often than reported for most human sarcoma types, with the exception of chondrosarcomas. In canine malignant lymphomas, the incidence of DNA-aneuploidy (21%) was much less than in the carcinomas / sarcomas mentioned above, which resembles the incidence in human Non-Hodgkin lymphoma, these lymphomas in both species considered similar entities. Yet, hypoploidy (1%) and the extend of the DNA-index deviation in hyperploid cases was smaller in the dog than in the human.

Conclusions: The higher rate at which hypoploidy or low level hyperploidy in dog cancers is apparently sufficient for manifestation of malignancy, as compared to similar cancers in the human, can be seen as evidence that the threshold of protection against carcinogenesis (aneuploidy evolution) is lower in the animal. It appears likely that the human (with risk 1/3 to develop cancer in a life-time of about 80 years) is better protected that animals such as dogs (similar life time risk, established in about 10 years). The lower incidence of aneuploidy in cancers such as lymphoma compared to carcinomas, and the seeming variations amongst different sarcoma types, may point to a variation of the influence of tissue structure and differentiation as restraints for cancer development, with varying levels of karyotype destabilization needed amongst different cancers and between species.

527 CDKN2A Promoter Hypermethylation is Associated With Environmental Risk Factors in Patients With Laryngeal Carcinoma
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Background: The rich landscape of small DNA variations is comprised of SNVs, insertions, deletions and structural alterations (CNVs) and structural variants (SVs) add another layer of genomic diversity. It is through sequencing and cataloging, in an unbiased manner, across the whole genome that the evolution of a tumor genome can be fully explored. The presence of aneuploidy, heterogeneity, tumor stromal contamination, and the like, makes the interpretation of cancer genomes even more challenging.

Materials and Methods: Complete Genomics sequencing platform [1], which produces 70mer paired-end reads, was used to generate tumor cell-line data to identify somatic mutations. Assembly was performed using the Complete Genomics Analysis Pipeline [2], which uses a hybrid approach of initial fast alignment to a reference followed by local de novo assembly in those regions of the genome that appear to contain variations. In recent literature, the SNP-level accuracy of the platform has been reported to be 94.4% [3] with a T/Tv ratio of 2.13 [3]. Comparative analysis was performed on both standard and high depths of coverage, which are minimally at 40x and 80x, respectively.

Results: Heterogeneity and aneuploidy impact the detection of small variants. To ameliorate these effects, the small variant caller models variable allele fractions rather than assuming diploidy in samples. Furthermore, consideration of triplod hypotheses enables improved resolution of somatic variations that are adjacent to germline mutations. When detecting CNV's and SV's, identification of nuanced events, such as copy neutral LOH is enhanced with the estimation of allele-specific copy number. Additionally, with high coverage, a 15% increase in relative sensitivity of het SNPs is observed at 40% tumor content.

Conclusions: The coupling of deeper sequencing with algorithmic enhancements has improved the sensitivity of detection of complex somatic events across a cancer genome. With multiple and often unknown etiologies, this landscape is very complex. Whole genome sequencing results in large datasets, associated with complexities of analysis and storage, stepping outside of the exome is indeed critical for detection of rare variants.

Reference(s)

529 Promoter Methylation of the Ligase IV Gene in Human Colorectal Cancer
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Background: Several genetic and epigenetic markers such as mismatch repair deficiency and methylation phenotype are used in diagnosis and prognosis of colorectal cancer. Yet, these markers are still insufficient to comprehensively explain the development and progression of these tumours. Here, we aimed to identify new epigenetic markers for colorectal cancer. As specific subgroups of genes such as DNA repair genes play an important role in these cancers, we focused on a detailed screen of DNA repair and repair-related pathways.

Materials and Methods: Methy-CpG immunoprecipitation (MeCP) followed by global methylation profiling on a CpG island microarray was used to
identify differentially methylated regions. These were quantitatively validated on bisulfite converted DNA by MassARRAY technology. DNAs from two independent colorectal cancer sample sets from Hong Kong (n=12) and Heidelberg (n=45) as well as 30 normal tissues were included. Differential methylation was further characterized for impact on mRNA expression by quantitative PCR after reverse transcription.

**Results:** MCP methylation profiles were obtained for 16 colon cancers. A repair gene-specific evaluation revealed 4 differentially methylated genes. Methylation status of 3 of the candidate genes was assured in the independent ethnic groups. The most promising candidate as it showed the strongest differential promoter methylation (up to 60% increase). Methylation coincided with down-regulation of LIG4 expression in 51% of cases. The functional consequence of LIG4 methylation was supported by reexpression of the gene after treatment of colorectal cancer cell lines with 5-aza-2'-deoxycytidine. As LIG4 is located on chromosome 13 which is frequently amplified in colon cancer, the locus was further tested in a subset of 47 cases for gene amplification by quantitative PCR. Comparison of amplification, methylation and expression data revealed that, in 28% of samples, the LIG4 gene was amplified and methylated, but expression was not changed. In our study population, methylation of LIG4 was not associated with known genetic and clinical markers of colon cancer.

**Conclusions:** Hypermethylation of the LIG4 promoter may represent a new epigenetic marker for colorectal cancer independent of known genetic or epigenetic markers. Currently, we are investigating the impact of promoter methylation both on cellular function as well as on its potential as a clinical marker of diagnosis and prognosis.

**[53] Evaluation of PTEN and PIK3CA Status in Breast Cancer for Patient Selection – Cross-validation Between Institutions**

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**Introduction:** PTEN and PIK3CA status are potential predictors of response to PI3K-pathway inhibitors. Therefore, we searched the most reliable platform to assess both PTEN expression and PIK3CA mutations in breast cancer (BC).

**Material and Method:** Cross-validation across platforms at SU2C sites was performed using three different sample sets: 18 formalin-fixed paraffin-embedded (FFPE) primary triple negative breast cancer (pTNBC) samples from VHUH/VWHO, 51 FFPE pTNBC samples from Columbia University and 21 FFPE all-comers BC from VHUH/VWHO. Moreover, we evaluated the transitions occurring between early and disseminated disease in 14 paired FFPE samples from VHUH/VWHO.

**Results and Discussion:** PTEN alterations were genotyped in 18 pTNBCs by OncoscanTM platform (Affymetrix) and were correlated to immunohistochemistry (IHC). PTEN loss of heterozygosity (LOH, n=4) or frameshift mutation (n=1) was concordant to PTEN protein loss (H-Score <50) by IHC in 4 samples; with the discordant LOH having an H-Score of 60. In the other two non-concordant samples (total of 3/18, 17% discordance), presence of non-tumor DNA, as assessed by genomicheterogeneity, impeded an accurate DNA copy number variation determination by OncoscanTM. PTEN protein loss by IHC was also cross-validated between two institutions. In a cohort of 12 pTNBCs containing eight PTEN low samples, only two samples were discordant (2/12, 17% discordance). Paired primary versus metastatic tumors were identified to transit either way, in the PTEN assessment by IHC (2/8 paired samples, 25% transition). PTEN IHC failed in most (3/5) metastatic bone samples due to Bouin’s fixative required for handling of this tissue. PIK3CA mutations by OncoscanTM were concordant to MassARRAY Sequencing (Sequenom) in 4 out of 5 mutated samples within the panel of 18 pTNBC. The discrepancy (1/18, 6%) was likely due to differences in the sensitivity of the two assays. In another cohort of 21 BC samples, PIK3CA mutational status was cross-validated by MassARRAY at two institutions (MDACC and VHUH/VWHO). Using clonal samples, we found that only two samples were discordant because of mutant allele frequency close to the sensitivity of the assay (10%). Manual review of the individual assays increased the concordance to 100%. Among 14 paired primary versus metastatic breast cancers we detected two transitions from wild type (WT) to H1047R mutation and one transition from E545K to WT (3/14, 21% transition), underscoring the need to determine PIK3CA status in metastatic lesions.

**Conclusions:** Evaluating both PTEN and PIK3CA status between institutions was due to cut-off and sensitivity of the assays respectively. For patient pre-screening purposes, MassARRAY and IHC can be performed at each SU2C site both in primary and metastatic breast cancer lesions. OncoscanTM is a valid, centralized platform for evaluation of PTEN and PIK3CA genomic alterations in BC.

**[52] The Tumor Suppressive Protein ING2 is Required for DNA Damage Response Proteins Recruitment and Promotes NHEJ**

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ING2 (Inhibitor of Growth 2) is a tumor suppressor gene whose expression is suppressed in breast, bladder, prostate and colon cancer, and melanomas. Furthermore, ING2 knock-out mice develop tumors spontaneously with a high frequency. ING2 belongs to the family of the ING proteins (ING1-ING5). Thus, ING2 is characterized by a Nuclear Localization Sequence (NLS) and a Plant HomeoDomain (PHD) motif in its C-terminal part. ING2 has been shown to bind to Histone H3 trimethylated on Lysine 4 (H3K4me3) via its PHD domain, allowing the anchorage of the deacetylase complex mSin3a/HDAC1 on the promoter of genes to regulate their transcription. ING2 is also a nuclear receptor of Phospholiposide 5 Phosphate (PI5P) which binds to the polybasic region (PBR) which is adjacent to the PHD. This interaction regulates the acetylation and thus the activation of the p53 tumor suppressor protein to induce senescence or apoptosis in response to genotoxic stress.

We have recently shown that ING2 ensures proper DNA replication and maintains genome stability. Thus, downregulation of ING2 as it occurs in human tumors, activates the intra S phase checkpoint observed by Cdk1 phosphorylation. It results in an accumulation of pH2AX, a marker of DNA double strand breaks (DSBs). Consequently, we have investigated the involvement of ING2 in the DNA Damage Response (DDR) pathway in response to DNA DSBs.

We propose that by regulating PHF16 expression, ING2 could be a new actor in the DNA DSBs signalling pathway. Thus, we describe a new mechanism by which ING2 may maintain DNA integrity.

**Reference(s)**


**[51] Investigation of an 11q13 Tumor Suppressor Locus in a Nasopharyngeal Carcinoma (NPC) Cell Line Identified NPA4 as a Candidate Tumor Suppressor Silenced by Promoter DNA Methylation in NPC and Other Tumors**

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**Background:** Aberrant promoter DNA methylation of tumor suppressor gene (TSG) is an important mechanism in carcinogenesis. In this ongoing study we report the identification of NPA4 as a potential TSG responsible for the tumor suppressive activity observed in a 1.8Mb minimally deleted region on chromosome 11q13 in NPC.

**Material and Methods:** Some of the major techniques used in this study include:

1. Semi-quantitative Reverse Transcription PCR
2. S′ RNA Ligase Mediated Rapid Amplification of cDNA Ends
3. Promoter Luciferase assays
4. Methylation Specific PCR
5. Bisulfite Genomic Sequencing
6. Real-Time PCR
7. Chromatin Immunoprecipitation
8. Variety of cell culture techniques

**Results:** NPA4 is expressed ubiquitously in a panel of normal tissue RNA but silenced or down-regulated in multiple carcinoma cell lines. The function of the promoter cradled in a CpG island was found to be methylated in tumor cell lines and primary tumor tissues and associated strongly with gene silencing. A predominantly epigenetic silencing mechanism was shown by pharmacological
demethylation induced by 5-aza-2'-deoxycytidine or genetic double knock-out of DNMT1 and DNMT3B which led to demethylation of the promoter and strong readacquisition of expression. NRP5A was further found to be up-regulated after Ultra-Violet (U) light irradiation and Adriamycin drug treatment and may be an indirect target of the TP53 tumor suppressor, suggesting a role in the DNA-Damage Response pathway. Ectopic expression of NRP5A in silenced cell lines elicits a strong inhibitory effect on cell clonogenesis, proliferation and migration.

Conclusions: Taken together, our data show that NRP5A4 could be one of the candidates TSGs responsible for the tumor suppressive function of NRP5A4 in silenced cell lines. Characterization of the mechanism by which NRP5A4 exerts tumor-suppressive effects is ongoing.

Introduction:

In this study it was aimed to determine whether or not p53 gene codon 72 polymorphism is a genetic marker of thyroid cancer development in multinodular goiter patients.

Material and Method: Genomic DNA was extracted from 90 persons (42 with multinodular goiter and 48 healthy controls) in the study. DNA was amplified with specific primers by PCR and RFLP technique was used to analyze p53 gene codon 72 polymorphism genotypes. PCR products were assessed with UV transilluminator by being exposed to agarose gel electrophoresis.

Results and Discussion: According to genotype distribution and allele frequencies of p53 gene codon 72 polymorphism there was no significant difference between groups.

Conclusion: As a result of our study we may assert that p53 gene codon 72 polymorphism cannot be considered as a genetic marker to develop thyroid cancer in Turkish population.

Analysis of the Codon 72 Polymorphism of P53 Gene in Patients With Multinodular Goiter to Develop Thyroid Cancer

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Conclusion: As a result of our study we may assert that p53 gene codon 72 polymorphism cannot be considered as a genetic marker to develop thyroid cancer in Turkish population.
Influence of the Polymorphism MTHFR C677T on the Development of the Colorectal Cancer

L. Delgado-Plascencia1, S. González-García1, V. Medina-Arana1, H. Álvarez-Aragüéez Cabrera1, E. Salido-Ruiz2, A. Fernández-Peralta3, J.J. González-Aguilera4, 5, University Hospital of Canary Islands, Surgery, La Laguna Tenerife, Spain, 2University Hospital of Canary Islands, Pathology, La Laguna Tenerife, Spain, 3Autonomous University, Molecular Biology, Madrid, Spain

Background: Colorectal cancer (CRC) is one of the most common cancers. The methylenetetrahydrofolate reductase (MTHFR) is a key-folate metabolizing enzyme, which catalyses the conversion of 5,10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate. The MTHFR C677T polymorphism is associated with the expression of a thermolabile enzyme with decreased activity that influences the pool of methyl-donor molecules. The 677T allele shows geographical variation in frequency. In this study, we carry out the analysis of the influence of the polymorphism MTHFR C677T on the development of the CRC in our population.

Material and Methods: We performed a retrospective study in which primary tumor samples from 50 patients diagnosed with sporadic CRC were collected at the University Hospital of Canary Islands, Spain. On the other hand, we obtained blood of the control group (103 patients). DNA from CRC cases were genotyped for MTHFR C677T by examining polymerase chain reaction-restriction fragment length polymorphisms produced with the Hinfl restriction enzyme.

Results: These two samples (cancer and control group) showed no significant departures from Hardy-Weinberg equilibrium (p = 0.37 and 0.58). CT and TT groups were most frequently observed in control group than cancer group (P = 0.04). When we considered CT and TT group together, this difference was increased, when compared control and cancer group (P = 0.01).

Conclusions: In conclusion, we found a correlation between the MTHFR C677T genotype and CRC. However, we did not observe relation between MTHFR C677T and histological features, so that we consider that MTHFR C677T can reduce the incidence of CRC, but as soon as the tumor appears, there are not differences about histological features.

Effect of the Polymorphism C677T Methylenetetrahydrofolate Reductase (MTHFR) in the Follow-up of Colorectal Cancer

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Polymorphism and Genetic Tendency to Colon Cancer

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Introduction: In this study we aimed to answer if Plasminogen activator inhibitor type-1 gene (PAI-1) gene 4G/5G polymorphism and Genetic Tendency to Colon Cancer

Polymorphism and Genetic Tendency to Colon Cancer

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Introduction: In this study we aimed to answer if Plasminogen activator inhibitor type-1 (PAI-1) gene 4G/5G polymorphism is a genetic mark of colon cancer in Turkish population.

Material and Method: Genomic DNA was extracted from 78 persons (39 with colon cancer and 39 controls). DNA was amplified by polymerase chain reaction using 4G allele- and 5G allele-specific primers. Products were assessed CCD camera by being exposed to %2 agarose gel electrophoresis.

Results and Discussion: According to genotype distribution 4G4G genotype frequency was statistically higher (p = 0.05) in the colon cancer group. The 4G allele frequency was indicated as 56% and 5G allele was as 44% in patients, whereas this was 46–54% in the control group.

Conclusion: This study has established that 4G/5G polymorphism genotypes of plasminogen activator inhibitor type-1 gene may be a genetic mark in the development of colon cancer in the Turkish population.

Polymorphism of the Enzyme Methylene tetrahydrofolate Reductase (MTHFR) C677T and Histological Findings in Colorectal Cancer

L. Delgado-Plascencia1, S. González-García1, V. Medina-Arana1, H. Álvarez-Aragüéez Cabrera1, E. Salido-Ruiz2, A. Fernández-Peralta3, J.J. González-Aguilera4, 5, University Hospital of Canary Islands, Surgery, La Laguna Tenerife, Spain, 2University Hospital of Canary Islands, Pathology, La Laguna Tenerife, Spain, 3University of La Laguna, Molecular Biology, La Laguna Tenerife, Spain, 4Autonomous University, Molecular Biology, Madrid, Spain

Background: Colorectal cancer (CRC) is the third most common cause of cancer-related death in the western world. It is widely known that histological features can influence in prognosis of this illness. On the other hand, several studies have observed a lower prevalence of CRC among individuals with the 677TT genotype. In this study, we carry out the analysis of the

Propagated May 2015 in colorectal cancer.
Results: Aberrant methylation of the BRCA1 promoter was detected in 33 of 241 (13.7%) unselected ovarian carcinomas. Methylation was more frequent in the BRCA1 mutation-negative (15.6%, 32/203) than in mutation-positive tumors (2.6%, 1/38, \( P = 0.0365 \)). Among the 38 mutation carriers examined, only one demonstrated aberrant BRCA1 promoter methylation. Methylation was more common in tumors with LOH at the BRCA1 locus (23.1%, 28/121) than in tumors with retention of heterozygosity (5%, 2/40, \( P = 0.0166 \)).

Aberrant methylation was observed in 3 of 13 (23.1%) undifferentiated, 3 of 17 (17.6%) other types, 24 of 169 (14.2%) serous, 2 of 18 (11.1%) endometrioid and none of 13 (7.7%) clear cell carcinomas. None of the 11 mucinous tumors had methylated BRCA1 promoter. There was no association between promoter methylation and serous histology. In addition, we found no differences in the frequency of BRCA1 methylation according to patient's age, disease stage or tumor grade.

Conclusions: Our findings suggest that promoter methylation may be an alternate mechanism of gene inactivation to mutation; in association with LOH, it may lead to biallelic gene silencing in sporadic cancers.

[544] Genomic Analysis of DNA Methylation and Copy Number Changes in Breast Cancer

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Breast cancer is the most common cancer type among women in the Western world with approximately one in every ten affected by the age of 70. Most patients have favorable disease outcome. There are, however, certain subtypes that are still difficult to treat and show significantly reduced survival rates. Further study is needed to get a better understanding of the genetic and epigenetic mechanisms behind these outcomes.

Results: This project aimed to identify novel markers for the early detection of breast cancer, as well as to better understand the mechanisms of resistance to chemotherapy. We analyzed DNA methylation and copy number changes in breast cancer using high-resolution genomic and epigenomic analyses of well defined samples with respect to subtype and clinical parameters. DNA methylation was analyzed in breast cancer samples and, where available, normal breast tissue from the same patient using Infinium 450K arrays. The array design interrogates >450,000 CpG sites including all RefSeq genes, their promoter regions without bias against those lacking CpG islands, as well as CpG sites outside of islands and protein-coding regions (Sandoval et al 2011). DNA copy number changes using high-resolution 385K aCGH design (Roche-NimbleGen) were compared to immunohistochemistry on tissue samples for subtype-specific markers (ER, PR, HER2, Ki-67, EGFR, CK5/6, CK8, CK16, MUC1 and Claudin-3) carried out on the same samples (Stefansson et al 2009; Stefansson et al 2011). This integrated approach is presented in clinical context with available data on histological grade, disease stage and breast cancer-specific survival outcomes.

Reference(s)

Sandoval et al 2011. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics, 6(6), 460-468.

[545] Downregulation of MiR-199b-5p is Associated With Chemoresistance Via Jagged1 Mediated Notch Signaling in Human Ovarian Cancer Cells

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Background: Ovarian cancer is one of the most deadly gynaecological malignancies worldwide. Adjuvant chemotherapy combined with other treatments is the current management of the advanced ovarian cancers. However, the acquisition of resistance to initially responsive tumors limits the effective curative rate. The underlying molecular mechanism remains largely unknown. Here, we report that the downregulation of miR-199b-5p leading to elevated JAG1-mediated NOTCH signaling activity may be involved in chemotherapy resistance in ovarian cancer.

Material and Methods: MicroRNA microarray (miR-199b-5p) expression was determined by Exiqon LNA™ microRNA microarray and quantitative RT-PCR (qRT-PCR). The demethylation treatments using 5-Aza-cdC and/or TSA were applied to show promoter hypermethylating scenario of miR-199b-5p in ovarian cell lines. Cell growth was assessed by XTT cell viability and colony formation assays. The computational softwares prediction, 3′UTR luciferase reporter and western blotting were used for miR-199b-5p. The −secretase inhibitor was used to inhibit Notch activity in ovarian cancer cells. Commercial siRNA and miRNA inhibitor were used for knockdown JAG1 and miR-199b-5p respectively.

Results: Using Exiqon LNA™ microRNA microarray profiling and q-PCR analyses, we found that miR-199b-5p was frequently downregulated in cisplatin resistant ovarian cancer cell lines. JAG1 was significantly associated with this downstage (\( P = 0.015 \)) and high grade (\( P = 0.003 \)) ovarian cancer samples (\( N = 31 \)). Intriguingly, the expression of miR-199b-5p could be restored upon treatments of 5-Aza-cdC and TSA in ovarian cancer cell lines (\( N = 5 \)). Functionally, re-expression of miR-199b-5p remarkably diminished cisplatin-resistance, whereas depletion of miR-199b-5p by siRNA approach augmented cisplatin-resistance in ovarian cancer cells. Computational prediction and western blotting identified JAG1, a significant Notch signaling effector, as a putative target of miR-199b-5p. Luciferase reporter assay using wild type or mutated miR-199b-5p targeting site at 3′UTR of JAG1 further demonstrated that miR-199b-5p could specifically repress JAG1 expression dose dependently. Importantly, enforced expression of JAG1 increased not only Notch signaling activity but also cisplatin-resistance in ovarian cancer cells. In contrast, knockdown of JAG1 by siRNA impaired the cisplatin-resistance of ovarian cancer cells. Furthermore, inhibition of Notch signaling by −secretase inhibitor showed the same effect in sensitizing ovarian cancer cells to cisplatin induced cell apoptosis, indicating that Notch signaling is involved in chemoresistance of ovarian cancer.

Conclusion: Our data suggest that the aberrant activation of JAG1/Notch signaling due to epigenetic silencing of miR-199b-5p may contribute to chemoresistance in human ovarian cancer cells.

[546] Mutation Screening of BRCA2 Exon 11 and Exon 17 in Bulgarian Breast Cancer Patients

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Background: Studies on different populations worldwide demonstrate that mutations in the BRCA2 gene are one of the two major causes of hereditary breast cancer. Protein-truncating mutations of BRCA2 are usually deleterious and increase the risk of breast cancer up to 80% over a lifetime.

Materials and Methods: We have screened 189 breast cancer patients, fulfilling the BCLC criteria, for germ-line mutations in BRCA2 in exon 11 and 17. 82 breast cancer patients for mutations in BRCA2 exon 17 Mutation analysis was performed by direct sequencing using 18 primer pairs. Results: In total 6 BRCA2 mutation carriers (3.2%) were found. The founder mutation described in Ashkenazi Jews 6174delT was observed in one patient with bilateral breast and ovarian cancer. The mutation was also found in the healthy daughter of the patient. A second deleterious mutation 5854delAGTT was detected in two patients with family history of breast cancer, one of them having the TNBC phenotype. The third mutation 1906delCT was detected in only one patient with early onset breast cancer. The only one mutation found in exon 17 2636delCT was detected in two members of one family, both of them having an early onset bilateral breast cancer.

Conclusion: The mutations discovered in the studied Bulgarian breast cancer patients were previously observed in other European populations. Further analysis of the other BRCA2 exons is necessary in order to ascertain the full spectra of the BRCA2 mutations in the Bulgarian breast cancer patients.

[547] MiRNA Regulation of FOXL2 Expression in Juvenile-type Granulosa Cell Tumours of the Ovary

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Background: Despite their distinct biology, granulosa cell tumours (GCTs) are currently treated similarly to other ovarian tumours. Predominantly expressed in granulosa cells, the transcription factor Forkhead Box L2 (FOXL2) is mutated in juvenile-type GCTs. However FOXL2 expression is near absent in juvenile-type GCTs. The aim of this research was to investigate the involvement of miRNAs as a mechanism of suppression of FOXL2 expression in juvenile-type GCTs. Materials and Methods: Luciferase assays were performed to determine if miRNAs bind to the 3′UTR of the FOXL2 mRNA in 3′UTR luciferase construct in COV434. Identified as promising candidates by miRNA microarrays, the miR-17 family was targeted for knockdown with the use of a miRNA sponge. In addition, the individual family members of the miR-17, 20, 92 cluster were knocked down using miRNA inhibitors. Subsequently, the effects on FOXL2 expression were assessed using RT-PCR and Western blotting. The miRNA sponge in combination with the 3′UTR luciferase construct was used to confirm the involvement of the miR-17 family in regulating FOXL2 expression in COV434 cells.

Results: Luciferase assays revealed that miRNAs bind to the 3′UTR of FOXL2 as indicated by at least a 50% decrease (\( p < 0.01 \)) in luciferase
activity compared to control treated cells. FOXL2 mRNA expression doubled (p = 0.037) at 24 h after the knockdown of the miRNA sponge, and had a four fold increase (p = 0.018) at 48 h. However, no increase in FOXL2 protein was observed. The knockdown of individual family members mir-17, mir-20b and miR-106a demonstrated that none of these miRNAs are directly responsible for the increase in FOXL2 expression observed with the miRNA sponge. However, when used with the miRNA sponge, the 3′ UTR luciferase construct revealed miRNAs were still able to bind the 3′ UTR of FOXL2.

Conclusions: It appears that the miR-17 family of miRNAs are able to regulate the expression of FOXL2 in an indirect manner, perhaps through altering the expression of genes upstream of FOXL2. Further studies are required to determine how the miR-17 family are regulating FOXL2 expression and what upstream genes they might be targeting.

**549 The Transcriptional Targets of Mutant FOXL2 in Granulosa Cell Tumours of the Ovary**

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Background: Despite their distinct biology, granulosa cell tumours (GCTs) are currently treated similarly to other ovarian tumours. Intriguingly, a recurring somatic mutation in the transcription factor Forkhead Box L2 (FOXL2) 402C>G has been found in nearly all GCTs examined. This finding that a single mutation may dominate a tumour’s development is unprecedented in cancer studies. The aim of this study was to identify the pathogenicity of mutant FOXL2 by studying its altered transcriptional targets.

Materials and Methods: Wildtype and mutant FOXL2 were overexpressed in the GCT cell line COV434, and total RNA hybridised to Affymetrix U133 Plus 2 microarrays. Comparisons were made between the transcriptomes of cells overexpressing wildtype and mutant FOXL2, to detect potential transcriptional targets of mutant FOXL2.

Results: The overexpression of mutant FOXL2 compared to wildtype FOXL2 led to altered expression of 425 genes (p < 0.1). Analysis using Ingenuity Pathway Analysis software indicated that the differentially expressed genes were enriched for functional annotations of tumourigenesis (p = 1.56 × 10−5), cell death (p = 2.70 × 10−5), and cell proliferation (p = 8.66 × 10−7). Furthermore, TGF-β1 signalling was found to be enriched when using the gene annotation tools GATHER (path:hsa04350, p < 0.001, Bayes Factor 7) and GeneSeDB (p < 0.001, False Discovery Rate of 0.19). This enrichment was still significant after performing a robust permutation analysis p < 0.01. This data concurs with data from the analysis of siRNA treatment of FOXL2 in the KGN cell line previously performed by us.

Conclusions: Given that many of the transcriptional targets of mutant FOXL2 are TGFB1 signalling genes, we speculate that deregulation of this key proapoptrotic pathway is one way mutant FOXL2 contributes to the pathogenesis of adult-type GCTs.

**550 RAF Fusion Transcripts Detected in Medulloblastoma but not Ependymoma Using RT-RQPCR Assays on RNA Isolated From FFPE Tissues**

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Introduction: Medulloblastomas and ependymomas are the most common malignant brain tumors in children, yet their molecular pathology remains unclear. There has been a significant increase in the incidence of these tumors in Iraqi children in the past two decades. Mutations in the tumour suppressor gene TP53 mutations have been implicated with the development of various cancers, but are rare in medulloblastomas and ependymomas. The occurrence and rate of TP53 mutations were analysed in Iraqi children diagnosed with these tumours in the last decade.

Materials and Methods: Tissue samples taken between 2006–2010 from 28 Iraqi children with medulloblastomas (22) or ependymomas (6). TP53 mutational analysis was performed by PCR amplification of exons 4–9, which harbor most of the mutations reported in various cancers, and direct sequencing. The investigation was performed in the laboratory of the Molecular Oncology Unit, Guy’s Hospital, London.

Results and Discussion: 21/28 patients (75%) had TP53 mutations. Inactivation mutations were detected in 15/28 patients (54%) (3/6 ependymomas (50%) and 12/22 Medulloblastomas (55%)). 7 patients (1 ependymoma and 6 medulloblastomas) had silent or intronic mutations. 2 medulloblastoma patients have multiple mutations. One patient showed a new mutation (a complex 13 exon deletion c.13181–13312 spanning part of intron 5-exon 6). It was interesting to note that 71/15 patients with TP53 mutations have the same mutation in exon 4 (c.412 C>G, P71R).

These data suggest that TP53 mutations are common in Iraqi children with medulloblastomas and ependymomas in present day Iraq. The rate of TP53 mutations in these patients is significantly higher than those detected in patients from other countries (up to 12% in reported studies), which may indicate different causes for the development of these tumours in these children.

Conclusions: These findings show TP53 mutations to play a more important role in the etiology of these tumours in Iraqi children than in those from other countries. This may point out to a possible impact of environmental factors experienced by mothers and children in Iraq in the last decade in Iraq, which require further investigation. The data may also indicate that medulloblastomas and ependymomas in Iraqi children have a different pathology and prognosis as compared to those detected in other countries, especially when considering that TP53 mutations in these tumours are associated with a resistant disease to chemotherapy.

**551 Genome-wide Unmasking of Epigenetically Silenced MicroRNAs in Hereditary and Sporadic Carcinomas**

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Introduction: MicroRNAs are evolutionarily conserved small non-coding RNAs predicted to negatively control the expression of up to one-third of messenger RNAs. MicroRNAs can serve as tumor suppressors or oncogenes and their expressions are remarkably deregulated in cancer due to multiple genetic and epigenetic alterations. DNA methylation of promoter regions is the best known epigenetic modification and can silence tumor suppressor and microRNA genes. Colorectal cancer develops through two main genetic instability pathways, one resulting from the defects in DNA mismatch repair system (MMR-D) and the other without any genetic defects in mismatch repair system, mismatch repair proficient (MMR-P). Here, we aimed to identify and epigenetically regulate microRNAs in hereditary and sporadic colorectal and endometrial cancer stratified by MMR status.

Materials and Methods: Three mismatch repair deficient (MMR-D) colorectal cancer cell lines (RKO, HCT116, HCT15), 2 mismatch repair proficient (MMR-P) colorectal cancer cell lines (SW480, T84) and 2 mismatch repair deficient endometrial cancer cell lines (HEC59, AN3CA) were treated with DNA methyltransferase inhibitor and histone deacetylase inhibitor followed by microRNA microarray analysis to identify epigenetically inactivated microRNAs. Tumor suppressor Methyltransfer-specific multiplex ligation-dependent probe amplification (MS-MLPA), TaqMan® Gene Expression, and immunohistochemistry analysis were performed to confirm the effect of drugs. Custom MS-
MLPA assay was developed to investigate promoter methylation of 5 chosen candidate microRNA loci on large patient series of hereditary and sporadic colorectal and endometrial cancers.

Results and Discussion: Twenty-four, 16, and 28 microRNAs with preferential involvement in MMR-D colorectal cancer, MMR-P colorectal cancer, and endometrial cancer, respectively, were significantly upregulated after the drug treatment as determined by microarray analysis. Five microRNAs (mir-129-2-5p, 375, 572, 663, and 483-5p) were chosen for further analysis based on the presence of the promoter CpG island and cancer-specific methylation as compared to the normal tissue. MS-MLPA studies of clinical samples are underway.

Conclusion: Epigenetic regulation of miRNA expression plays an important role in colorectal and endometrial tumorigenesis regardless of MMR status and may prove useful as diagnostic biomarkers in these disorders.

552 Polymorphisms Within MicroRNA Binding Regions and MicroRNA Expression Profiling as Biomarkers for Colorectal Cancer

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Colorectal cancer (CRC) results from the combined effects of environmental and genetic risk factors, with a crucial role of DNA repair pathways for CRC risk. We screened among SNPs residing in 3′UTRs of NER genes and simultaneously located in miRNA binding sites and calculated the binding free-energy according to all alleles. Finally, we tested 9 NER SNPs in a population of 1093 CRC cases and 1469 controls from the Czech Republic. Rs7356 in RPA2 and rs4596 in GTF2H1 were associated with CRC risk, in particular with rectal cancer (OR 1.52, 95% CI 1.02-2.26, p = 0.04 and OR 0.69, 95% CI 0.50-0.94, p = 0.02 resp.). Variation in miRNA target binding sites of NER genes may be important for modulating CRC risk, with a different relevance according to tumor location. Alterations in miRNA expression profiles have recently been detected in various human tumors, including CRC. Accumulating evidence shows that miRNA expression patterns are unique to certain cancers and have potential as early diagnostic biomarkers as well as prognostic and predictive factors.

In our investigation, we found that 23 miRNAs were differentially expressed in normal and rectal tumor tissues. In particular, miR-486-5p, previously not described, resulted overexpressed in tumors (p < 0.0002). This is one the first study describing miRNA expression profiling specific for rectal cancer highlighting again the peculiarity due to tumor location. Acknowledgements: GACR grants P304/10/1288 and P304/12/1585.

553 Histone Modification Enzymes Predict Clinical Outcome in Colorectal Cancer

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Introduction: Current colorectal cancer (CRC) staging according to the TNM system (AJCC) is insufficient to accurately predict clinical outcome. Therefore, there is a great need for new prognostic biomarkers. Epigenetics is a rapidly developing field in cancer research and has the potential to identify new diagnostic and prognostic biomarkers. In addition to DNA methylation, histone modifications are key epigenetic regulators of gene transcription. Sirtuin-1 (SIRT-1) is a histone-deacetylase that specifically targets acetylation of the Lysine 9 residue on histone H3 (H3K9ac). The results from the exon-specific expression arrays show a deletion in the regulatory region of the gene. The deletion includes more than a thousand of adenomas in the large bowel and untreated carriers inevitably develop colorectal cancer (CRC) at an early age. A milder form of FAP also exists referred to as attenuated polyposis (AFAP). The APC gene (5q21-q22) encodes several tissue specific transcripts and two promoter regions have been identified 1A and 1B. In Family 1 of the Swedish Polyposis Registry we have identified a large novel deletion in the regulatory region of the gene. The deletion includes more than half of the promoter 1B. Q-PCR results indicate markedly lowered levels of expression from promoter 1B in mutation carriers. Unexpectedly in blood, elevated levels of expression from promoter 1A were observed. This higher expression was not seen in samples from normal colon mucosa or in adenoma from mutation carriers (Rohlin et al. 2011). In adenomas no inactivating mutation could be detected (Rohlin et al. 2011). Our findings indicate that alternative pathways, besides the governing wnt-signaling, may be involved in tumor initiation in FAP patients.

In order to investigate the contribution of different pathways we examined if 94 key tumor suppressor genes and oncogenes might be affected in patients harboring germline mutations in the APC gene and the effect on the expression of these genes in blood, normal colon mucosa and adenoma tissue from the same patient.

Material and Methods: Blood, normal colon mucosa and adenoma tissue from patients in Family 1 and from 4 other APC mutation positive patients were analysed by exon-specific expression arrays (GeneChip®Exon 1.0 ST AFAP expression Array) and wnt-signaling and oncogene- and tumour-suppressor pathway PCR arrays (SABiosciences RT™ profiler™ PCR array).

Results: The results from the exon-specific expression arrays show a significant higher expression of the CDH1 gene in blood from mutation carriers in Family 1 compared with normal controls. Several genes in different pathways indicate up- and down regulation in the different tissues from patients with germline mutations in the APC gene.

Conclusions: Differences in gene expression in blood compared with normal colon mucosa and adenomas tissue can add information regarding which pathways and genes might be involved in tumor initiation in FAP patients.

555 CTCF and the Global Effects of Distinct Histone Marks on BCL6 Expression Pattern of Oncogenes and Tumor Suppressor Genes in APC Mutation Positive FAP Patients

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Introduction: Familial adenomatous polyposis (FAP) is caused by dominant inheritance of germline mutations in the adenomatous polyposis coli (APC) tumor suppressor gene. In classical FAP mutation carriers develop hundreds to thousands of adenomas in the large bowel and untreated carriers inevitably develop colorectal cancer (CRC) at an early age. A milder form of FAP also exists referred to as attenuated polyposis (AFAP). The APC gene (5q21-q22) encodes several tissue specific transcripts and two promoter regions have been identified 1A and 1B. In Family 1 of the Swedish Polyposis Registry we have identified a large novel deletion in the regulatory region of the gene. The deletion includes more than half of the promoter 1B. Q-PCR results indicate markedly lowered levels of expression from promoter 1B in mutation carriers. Unexpectedly in blood, elevated levels of expression from promoter 1A were observed. This higher expression was not seen in samples from normal colon mucosa or in adenoma from mutation carriers (Rohlin et al. 2011). In adenomas no inactivating mutation could be detected (Rohlin et al. 2011). Our findings indicate that alternative pathways, besides the governing wnt-signaling, may be involved in tumor initiation in FAP patients.

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Conclusions: Differences in gene expression in blood compared with normal colon mucosa and adenomas tissue can add information regarding which pathways and genes might be involved in tumor initiation in FAP patients.
and supporting chromatin interactions and in the epigenetic regulation of a number of genes.

Material and Methods: Gel retardation and chromatin immunoprecipitation (ChIP) assays were performed to study the CTCF binding to the BCL6 regulatory region. The presence of histone marks upon silencing of CTFC was analyzed by ChIP. Protein and mRNA levels were analyzed by western blot and qRT-PCR, respectively.

Results: We identified a CTFC binding site in exon 1A of BCL6 within a CpG island, which is unmethylated in cell lines and primary lymphoma samples. CTFC binding site and occupation in vivo to the BCL6 regulatory region was observed. Variant histone H2AZ and marks of active chromatin such as H3K4me2 and H3Ac, are present at the CTFC binding site in BCL6 expressing lymphoma cells. On the contrary, much lower chromatin enrichment was found for repressive histones, H3K9me3 and H3K27me3. Silencing of CTFC with a shRNA vector reduces BCL6 mRNA and protein levels. These changes were accompanied with loss of active histone marks and occupation of repressive marks at the BCL6 exon 1A regulatory region.

Conclusion: spearheaded by silencing of BCL6 regulatory region against repressive histone marks and induces chromatin modifications at the BCL6 locus. Overall we have shown that CTFC binds to a novel site within the exon 1A of the transcriptionally active BCL6 locus and may contribute to maintain transcription.

A Demethylation Screen Identifies Zinc Finger Protein silencing of Vik-1 and to explore the value of Vik-1 as a potential biomarker in lung cancer. Very little is known of the function of Vik-1, but the protein has some properties consistent with a potential tumour suppressor function. Work is ongoing to elucidate the functional consequences of this transcriptional silencing of Vik-1.

Background: The importance of epigenetic transcriptional silencing as a mechanism of inactivation of genes encoding putative tumour suppressor functions is well recognised. The objective of this project was to seek de novo, candidate lung cancer tumour suppressor genes that are subject to transcriptional silencing.

Methods: Genome-wide methylation analysis was performed using methylation microarrays (Human 450K Methylation BeadChip) and methylation reversal assays with 5-aza-2-deoxycytidine treatment coupled with whole genome mRNA (Illumina HumanHT-12 v4 Expression BeadChip) in a panel of lung cancer cell lines. Methylation of candidate genes was validated in a panel of 21 lung cancer cell lines (16 non-small cell lung cancer, 6 small cell lung cancer) and a series of 134 lung cancers using pyrosequencing and methylation-specific PCR (MSP). To assess the functional consequences of Vik-1 methylation, Vik-1 was ectopically expressed using an expression vector or transduction with a retrovirus.

Results: We identified the transcription factor Vik-1 (Vav-interacting kinase 1; ZNF655) as a novel gene subject to methylation-dependent transcriptional silencing in lung cancer. Differential expression was confirmed in a panel of 21 lung cancer cell lines and 10 non-tumour primary lung tissue samples using RT-PCR and quantitative PCR. Vik-1 mRNA was detectable in 10/10 non-tumour lung tissues analysed, but was absent in 2/21 lung cancer cell lines and could be reactivated by azacytidine in such cell lines. Pyrosequencing revealed dense methylation in the CpG island located in the 5′ regulatory sequences of the Vik-1 gene, and this correlated with absent or down-regulated expression. Methylation was confirmed by MSP: Using pyrosequencing and MSP, methylation in the Vik-1 CpG island was detected in 18% of lung cancer tissue samples obtained during surgical resection from patients with newly diagnosed non-small cell lung cancer (NSCLC).

Conclusion: Our data show that Vik-1 is subject to epigenetic silencing in lung cancer. Very little is known of the function of Vik-1, but the protein has some properties consistent with a potential tumour suppressor function. Work is ongoing to elucidate the functional consequences of this transcriptional silencing of Vik-1 and to explore the value of Vik-1 as a potential biomarker in lung cancer.

The Genomic Landscape of ER Positive, HER2 Negative Breast Cancer

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Background: Estrogen receptor positive (ER+), Human Epidermal Growth Factor Receptor 2 negative (HER2−) breast cancer is the most common form of breast cancer occurring in nearly 40% of all known cases. The Breast Cancer Somatic Genetic Study (BASIS) was established as part of the International Cancer Genome Consortium (ICGC) to comprehensively catalogue somatic mutations in 500 ER+HER2− cancers using next-generation sequencing technology. The work presented here will showcase some of the recent and novel findings from an analysis of a subset of 70 such breast cancers.

Material and Methods: DNA and RNA samples for this study were obtained from various collaborators of the BASIS project in Europe, UK and U.S.A. For each tumour sample, a matching lymphocytic DNA sample was also collected to enable tumour-normal comparisons during data analysis. These samples also have detailed clinical data available. Relevant institutional ethics committees approved the accrual and use of patient samples and information related to this project.

All tumour-normal pairs were subjected to standard genotyping, quality control and library preparation methods. All DNA sequencing was performed on Illumina HiSeq® machines by the sequencing core at the Sanger Institute. It was ensured that each tumour sample had ~40 and each normal sample had ~30 times genomic coverage.

Each tumour-normal pair was analysed for single base changes (substitutions) using the in-house algorithm CAVE MAN, multi-base genomic insertions and deletions using the published algorithm PINDEL and structural variants using the in-house algorithm BRASS. Changes across multiple samples were assessed together to identify recurrent, novel somatic mutations.

Results: Preliminary results suggest that high-depth, next generation sequencing data has the ability to reveal novel somatic mutations in both coding and non-coding regions of the genome. Understanding the functional consequences of these novel mutations will increase our current knowledge of the ER+HER2− breast cancer genome. These discoveries might also enable better diagnosis and prognosis of patients.

Conclusions: This study highlights the power of large cohort studies and of new technologies to provide us with a greater understanding of a disease whose genetic landscape is yet to be fully mapped.

LINE-1 Hypomethylation Predicts Tumor Recurrence and Patient Survival in Early Stage Rectal Cancer

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Background: In rectal cancer, there is a need for biomarkers predicting clinical outcome as the current TNM staging system (AJCC) leads to both over- and undertreatment. Epigenetics is a promising field of cancer biomarker research since deregulation of gene expression is a major factor in tumorigenesis. Because of their abundance in the human genome, methylation of non-coding repetitive sequences such as long interspersed element 1 (LINE-1) and Alu repeats has been used as surrogate marker for global methylation. LINE-1 has retrotransposon activity, and methylation of this sequence has been shown to be a significant prognostic biomarker of disease outcome in different cancers. In this study, we evaluated LINE-1 and Alu methylation in a panel of 218 rectal cancer formalin-fixed paraffin-embedded (FFPE) tissues and 73 normal FFPE tissues obtained from patients enrolled in the Dutch total mesorectal excision (TME) clinical trial.

Materials and Methods: DNA was extracted from two 7 μm FFPE tissue sections per patient and treated with sodium bisulfite. LINE-1 methylation was assessed using quantitative real-time PCR using universal primers and methylation specific LNA probes. A pilot study was performed comprised of 28 tumor tissues (stage I/II) and 25 normal tissues. A validation study was performed including 190 tumor (stage I/IV) and 48 normal tissues. Statistical analyses were done using the Cox proportional hazard model and Kaplan-Meier curves.

Results and Discussion: In early stage rectal cancer (stage I/II), multivariate analyses showed that hypomethylation of LINE-1 was an independent prognostic factor, showing a significantly worse survival (p=0.001; HR 5.2) and a higher chance of recurrence (p=0.017; HR 9.7). Alu methylation was relatively stable and was not correlated with tumor stage (p=0.601) or clinical outcome (overall survival; p=0.650). Therefore, it is possible that changes in methylation of LINE-1 reflect an active involvement of LINE-1 in rectal cancer tumorigenesis, rather than representing a global methylation status. This needs to be further investigated.

Conclusion: LINE-1 methylation can be used as a biomarker predicting survival and recurrence in rectal cancer patients complementing the current staging system. This can be used to determine treatment strategies for individual patients, thereby preventing over- or undertreatment.
A New Molecular Inversion Probe (MIP) Based and Cancer-focused Whole-genome Copy Number Platform Requires Only 75 Ngg of Input DNA From FFPE Samples

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Background: Copy number (CN) and somatic mutation studies of cancer are atherapizing challenge for discovering reliable biomarkers of cancer that can predict clinical outcomes. We have shown that the Molecular Inversion Probe (MIP) assay used in the OncoscanTM platform works well with thousands of degraded FFPE samples [1]. In collaboration with leading cancer researchers, the coverage of the existing platform was further enhanced by supplementing the probe panel with new probes to cover an additional 200 cancer-relevant genes. The OncoscanTM FFPE Express 2.0 deliver three types of data (SNP, CN, and somatic mutation) from just 75 ng non-amplified genomic DNA.

Method and Design: New MIP probes were designed to target the 200 cancer relevant genes and provide: (a) greater marker density; (b) high MAF (minor allele frequency) to enable SNP analysis; (c) good quantitative performance. The platform employs a QC matrix MAPD (mean of absolute pairwise difference), for assessing data quality [2].

Results and Discussion: The median probe density increased from 1500 to 5000 bins per gene in microarrays and from 3500 to 15000 bins in oncogenes. The performance of additional probes were assessed in the following categories:

1. Geying – the average call rate for 48 Hapmap samples with 334K passing SNPs was 98.82%; for normal FFPE, 99.78%. The average accuracy for 48 Hapmap was 99.38%.
2. Copy number – probe reproducibility was measured at CN=1, 0.1 and 2. The average reproducibility of copy number changes was >95% as measured at the probe level in the SNPs that reside in the 200 genes. 97% of the newly designed probes passed these criteria. The dynamic range for copy number determination was assessed with BAC clone spin-in.
3. Somatic mutation analysis was tested in cell lines with known mutations, including EGFR_pE746_A750del, KRAS_pG12D/V, PIK3CA_pE545K and PIK3CA_pH1047. Concordance of 100% was observed from six repeats of each mutation; testing was performed in two different laboratories. The false positive rate was 0.63% when assessing performance with normal samples.

Conclusion: The content of the OncoscanTM platform was expanded to include 200 genes of high value in cancer research and diagnosis. The platform was shown to work well on both fresh and archival FFPE samples. SNP CN, and somatic mutations can be interrogated by the same assay, offering an unprecedented opportunity for both retrospective studies where only FFPE samples are available and for ongoing clinical research where only tiny amounts of biopsy are accessible.

Reference(s)

Evaluation of Cytotoxicity and Genomic Instability of Bixin on Human Leukemia HL60 Cell Line

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Bixin is a carotenoid extracted from annatto (Bixa orellana) seeds, one of the largest sources of natural red pigments, which has beneficial antioxidant properties. This study aimed to evaluate bixin cytotoxicity and genomic instability on human leukemia HL60 cell line. For this purpose, cytotoxicity was measured by MTT assay and micronucleus test and comet assay. For MTT test, HL60 cells were treated with concentrations of bixin from 0.01 to 5.0 µg/mL and, for micronucleus and comet tests were treated with bixin 0.03, 0.05 and 0.08 µg/mL. Untreated cells were used as negative control. The micronucleus was verified in 2,000 binucleated cells per treatment. The comet assay parameter used was %tail DNA and was examined in 100 nucleoids per treatment using Cometscore®. Bixin-induced cytotoxicity increased in a concentration-dependent manner. The treatment at concentrations from 0.01 to 0.1 µg/mL showed cell viability above 80% while treatment with 5.0 µg/mL showed only 22%, significantly different from negative control (p<0.05). Three bixin concentrations with cell viability above 80% were analyzed for their possible genotoxicity by Comet assay. The result was not observed a significant increase of genomic instability. According to these results, we suggest that bixin did not present genomic damage in our experimental conditions, although it has been cytotoxic at high concentrations.

Regarding crucial relevance of bixin for human health and commercial uses, further studies have been carried out in our laboratory. Financial support: CNPs.

Evaluation of the MiRNA Expression Profile in Human Upper Gastrointestinal Cancers Associated With Environmental Factors in Northwest Hungary

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Introduction: Esophageal and gastric cancers are among the most common cancer-releated death worldwide. Their geographical distribution is very diverse. It is known that the expression of miRNAs, as a new class of non-coding small RNAs, is very sensitive to environmental exposure. The aim of this study was to investigate the correlation between the regional distribution of patients with esophageal and gastric cancers and their miRNA expression pattern in Northwest Hungary.

Materials and Methods: The geographical distribution of 113 cases with esophageal and gastric cancer obtained from Oncoradiology Center of Szombathely were illustrated using geographic information system. Further epidemiological evaluation were performed according the patients clinicopathological data. 48 formalin fixed paraffin embedded primary cancer samples were chosen for further analysis of miRNA expression level including miR-21, miR-27a, miR-34a, miR-93, miR-143, miR-155, miR-196a, miR-203, miR-205, miR-221, miR-223.

Results and Discussion: The incidence of gastric adenocarcinoma was approximately two times higher than esophageal squamous cell carcinoma. More than 90% of the cases with gastric and esophageal cancer had a positive anamnesis for alcohol consumption and smoking. The miRNA expression of patients with esophageal cancer was significantly different among smokers and non-smokers. In the ESCC samples obtained from smokers the expression level of miR-21 (p=0.032), miR-34a (p=0.037) and miR-155 (p=0.045) were markedly higher than in non-smokers, while an opposite tendency was observed in the expression of miR-203 and miR-205. Investigating the impact of living environment on the miRNA expression in gastric cancers, we found significantly higher level of miR-203 and miR-205 (p=0.043 and p=0.0407) in samples originating from patient living in villages than in cases of bigger cities. Our study showed that altered expression of miRNAs esophageal and gastric cancers could be related to the existence of common risk factors such as smoking or drinking water conditions.

Conclusion: Analysis of miRNA expression profile can indicate the importance of the environmental factors in the pathogenesis of the upper gastrointestinal cancers and contribute to the deeper understanding of their role in the human carcinogenesis.

Early Aberration of MicroRNA Expression Profile After Treatment With MN

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Introduction: N-methyl-N-nitrosourea is a direct-acting DNA alkylating carcinogenic agent. There is emerging evidence concerning the role of miRNAs in response to environmental carcinogens by regulating gene expression. The aim of this study to investigate the in vivo effect of MN on the level of miRNAs include miR-21 oncomiR, miR-146a playing are involved in NFkB antiapoptotic pathway and let-7a functions as a tumor suppressor targeting ras and c-myc genes, in order to reveal their early effect in chemical carcinogenesis as biomarkers.

Materials and Methods: The expression level of miR-21, miR-146a and let-7a was investigated in CBA/CA H2O inbred mice 24 hours and one week after MN treatment (single dose of 30 mg/kg body weight) using real-time PCR methods.

Results and Discussion: According to our results the MNU treatment significantly increases the expression of miR-21 in all investigated organs compared to the controls. Furthermore in the lungs we detected more than two times higher level of miR-21 one week after exposure than 24 hours after the same chemical exposure. In the spleen the expression of all investigated miRNAs were found to be significantly increased by 1.93–10.47 fold change in both treated groups compared to the controls. Except of the spleen, MNU treatment caused a reduction of miR-146a expression compared to the non-treated groups. There was no significant alteration detectable in the lungs 24 hours after the MNU administration, while one week after the treatment the miR-146 expression was more than ten times lower than in the control group. The miR-146a gene expression also showed markedly reduction in the kidney, whereas found more times lower level on the 7th day and six times lower level at the 24-hour time point in relation to the controls. The elevated level of miR-21 after MNU treatment indicates that there is a link between the overexpression of miR-21 and carcinogenesis. The
overexpression of let-7a, mir-21 and miR-146a in the spleen can refer to the fact that MNU induce tumorigenesis in the hematopoietic system.

Introduction: The acquisition of somatic alterations (point mutations/ chromosomal rearrangements) underlies the hallmarks of cancer, generating genetic diversity that drives tumorigenesis. Advances in the study of cancer genomes revealed in solid tumors a complex pattern of copy number alterations (CNA), structural rearrangements, and aneuploidy. Breast cancer (BC) is the most common malignancy in females, being the leading cause of death by cancer. This heterogeneous disease is not fully understood yet, however, genomic studies have identified unique CNA patterns in different BC subtypes. Regarding the subtype triple-negative (TN; estrogen and progesterone receptors, and HER2 negative) expression levels, only limited data are available which genes or chromosome regions are involved in its initiation and progression.

Material and Methods: We assessed the genomic profile of CNA in 16 triple-negative breast carcinomas (TNBC), aiming to identify genomic markers related to lymph node status that could be of clinical importance. The array-CGH survey were conducted on primary TNBC samples, obtained from patients with (8) and without (8) lymph node metastasis at diagnosis. Data was obtained using a 60k oligoarray (OGT), with average probe spacing ~50kb, and analysis was performed on the Software Nexus 6 (Biodiscovery).

Results and Discussion: The most prevalent chromosomal alterations were losses at 3p, 4q, 5q, 6p, 13q, 18, and 21q; additionally, gains on 1q, 3q, 8q, 10p, 17q & 19w were frequent events. However, although these CNA have been detected in >50% of all samples, most of them exhibited low log, ratios values, indicating that they were present in mosaic. Notably, TNBC from patients with lymph node metastasis exhibited a slightly distinctive pattern of genomic alterations, mainly characterized for a significantly increased frequency of low amplitude losses at 13q and 18q, and gain at 1p. The most relevant CNA associated with TNBC for lymph node metastasis was the gain of a small segment at 19p13.11 (66kb), harboring at least 20 RefSeq genes (gain detected in 5/8 positive for 19p13.11 and 0/8 for lymph node metastasis). Conclusion: The recurrence of specific CNA in TNBC indicates sites likely harboring genes whose copy number changes favors neoplastic progression. Therefore, recognizing specific patterns of genomic alterations associated to lymph node status may be relevant in primary TNBC can provide clues on the mechanisms driving its progression. In a progressive model of tumor evolution we can propose a link between the somatic acquisition of the 19p13.11 gain in TNBC and the occurrence of lymph node metastasis. It is interesting to mention that a recent study (Stevens et al, Cancer Res 2012) discloses a 19p13.11 SNP variant associated with risk of TNBC; our finding reinforces the role of this genomic region for this BC subtype.

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555: MI RNA Expression Patterns of Colorectal Adenocarcinomas

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Introduction: Recent advances in the field of RNA research provided evidence implicating non-coding RNA molecules in colorectal carcinogenesis. Epigenetic alterations in colorectal cancer that colon tumor epithelial cells into adenocarcinoma cells include aberrant expression of miRNAs. We customized and tested a panel of miRNAs for human colorectal adenocarcinoma.

Material and Methods: colorectal adenocarcinoma and autologous normal colon epithelial samples (No. 56) were obtained during colonoscopy from patients diagnosed at Pécs University Surgery Clinic. After histopathological evaluation tumors were analyzed in LightCycler 480 PCR systems for the expressions of 10 hsa-miRNAs (miR-21, miR-27a, miR-34a, miR-143, miR-146a, miR196a miR-155, miR-203, miR-205, miR-221) compared to 5 SS RNA and U6 snRNA as internal controls. Expression profiles were ordered and statistical significant evaluated according to clinical stage, histological grade and cancer location.

Results and Discussion: Expression pattern differences of the investigated miRNAs showed statistically significant correlations with the clinical stage and histology grade. High Grade T4N1M1– T3N0M0 tumors as well as the low grade T2N0M0, T1N0M0 adenocarcinomas showed significant and characteristic over-expression compared with the autologous normal according to miR-21, miR27a, miR-34a, miR155 and miR221 in paired two-sample t-tests. Mir-146a and 196a were specific only for adenoma samples. Analysis of variance (ANOVA) of the significantly up-regulated miRNAs (miR-21, 27a, 34a and 155) was also found to be significant matching high grade tumors with low grade tumors (p: 0.03 CI: 3.75–4.49) and high grade adenoma (p: 0.019 CI: 1.744–3.362) tissues. A correlation between adenocarcinoma location and mir-155 expression was also observed, but it reached no statistical significance on the analysis.

Conclusions: miRNA profiling is a suitable method for molecular charac-
terization of colorectal adenocarcinoma, standing out as a potential new biomarker. Determining the causes and roles of epigenetic regulation in colorectal adenocarcinoma will lead to effective prevention and therapeutic strategies for colorectal cancer patients.

556: HOXA9 Target Genes in Glioblastoma – Characterization and Clinical Relevance

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Background: Gliomas, originating from the predominant glial tissue, are the most common primary tumors of the central nervous system (CNS) in adults. The most common form is glioblastoma (GBM, WHO grade IV), and the median survival of the affected patients is approximately 1 year after diagnosis. Some putative prognostic biomarkers in GBM patients include MGMT methylation and HOXA9 expression, but the clinical significance of the current panel of biomarkers is imperative. Overexpression of HOXA9 is associated with pro-proliferative and pro-invasion properties and with a poor prognosis. As HOXA9 is a transcription factor, we hypothesized that a set of HOXA9-transcriptionally regulated genes may be its true biological effectors. In this sense, we aimed to characterize the HOXA9 transcriptome in GBM to identify novel prognostic biomarkers and therapeutic targets.

Material and Methods: We performed expression microarrays in HOXA9-negative and HOXA9-overexpressing U87MG cells, and the resulting transcriptome was studied using bioinformatics tools. In order to validate the microarray results, a subset of the over- and under-expressed genes were tested by reverse-transcriptase polymerase chain reaction (RT-PCR). To verify which of the differentially expressed genes are direct targets of HOXA9, an in silico analysis was performed. By RT-PCR we inferred a few of these putative HOXA9 direct targets in GBM cell lines (A172, which endogenously expresses HOXA9 and U87-MG (retrovirally infected to overexpress HOXA9, and the respective MSCV empty control vector). Chromatin immunoprecipitation (ChIP) was performed in U87MG-HOXA9, U87MG-MSCV and A172 cell lines to evaluate the direct binding of HOXA9 to the promoter regions of the newly-identified targets. As HOXA9 expression has prognostic value in GBM patients, we also evaluated if these HOXA9 targets are associated with patients’ survival in two different cohorts of patients (TCGA and Rembrandt databases).

Results: By gene expression array analysis, we found 1537 genes upregulated and 1917 downregulated in U87MG-HOXA9 cell line compared to U87MG-MSCV. The functional clustering analysis revealed several cancer-related pathways upregulated in HOXA9+ cells. RT-PCR analysis to a small subset of those cancer-related genes validated our array data. Chip experiments showed that HOXA9 transcriptionally activates some of these genes by directly binding to their promoter regions. Finally, the overexpression of several HOXA9-regulated genes was associated with a worse survival in two independent sets of GBM patients.

Conclusion: Our study provides the first characterization of HOXA9 target genes in the context of GBM, and identifies new clinically-relevant prognostic biomarkers. Our findings may help to rationalize therapy decisions and to identify new molecular targets for therapy.

557: Effects of Procont on miRNA Expression of Let-7, MI-21 and MI-146a

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Background: Procon is a potential chemopreventive dietary supplement that exerted significant tumor suppressive effect in animal carcinogenicity bioassays. Genomic analysis showed suppression of Ha-ras, K-ras and c-myc in vital organs of CBA/Ca mice when Procon was administered in combination with 7, 12 dimethylbenz[a]anthracene (DMBA) an indirect carcinogenic agent. There is emerging molecular evidence concerning the modification of miRNA expression patterns after treatment with chemopreventive agents. We investigated the impact of Procon on let-7a, miR-21, and miR-146a expressions.

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Materials and Methods: 6-week-old CBA/CAH2 haplotype mice of both sex were used. The first group was the control group where animals consumed the standard laboratory chow pellet. The second group received intraperitoneal gavage of DMBA in 20 μg/dose at the start of the examination. The third group consumed Procont for 7 day before the exposure of DMBA. The expressions of let-7a, miR-21 and miR-146a were determined in the liver, spleen, lung and kidney tissues of animals using LightCycler 480 PCR System.

Results and Discussion: The DMBA exposure resulted increased expression of the let-7a gene in 17 out of the 25 samples. These results showed a marked change in all examined tissues compared to the controls. Based on our data, dietary administration of Procont before carcinogen exposure induced a very characteristic decrease in the expression level of let-7a, miR-146a and miR-21 after just 7 days consumption in mice fed by Procont compared to those receiving no treatment or DMBA alone. MiR-146a level in kidneys was detected to be more than six times higher in DMBA treated group than in mice consumed Procont before DMBA gavage. We observed the lowest expression of let-7a in spleen and lung tissues at the DMBA exposed mice treated with Procont. The miR-21 and miR-146a gene expression also showed marked reduction in mice lung relative to the other organ. Especially the expression of miR-21, where we found nine times lower expression.

Conclusions: Procont presents as a potential chemopreventive dietary supplement that induces demonstrable expression alterations on both miRNA and mRNA level. We have several undergoing experiments that clarify the chemopreventive effects of Procont.

[557] Genomic Analysis of Non-Hodgkin Lymphomas Reveals Mutations in Chromatin Remodeling Genes

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Introduction: Follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) are the most common types of non-Hodgkin lymphomas (NHLs). Differential gene expression profiles are used to classify subtypes of DLBCL and specific gene expression signatures reveal unique diagnostic and prognostic information in DLBCL and FL.

We recently reported the identification of more than 100 genes that are recurrently inactivated or activated by point mutation in NHLs, but our grasp of the full scope of mutation including CNVs and SNVs affecting splice sites, unexpressed transcripts and non-coding regions of the genomes remained incomplete.

Materials and Methods: To capture such mutations and determine their significance in DLBCL, we have sequenced the tumor and matched normal DNA from 56 patients and 56 matched normal DNA. The majority of these mutations are truncations and frame-shifting indels, inactivating MLL2. These mutations may result in abnormal chromatin marks, consequently switching genes from transcriptionally active to silent, or vice versa. MLL2 was somatically mutated in 4% of FL (n=35) and 32% of DLBCL (n=37). The majority of these mutations are truncations and frame-shift indels, inactivating MLL2. Approximately half of the mutated samples have two independent mutations in MLL2. In cases where allelic discrimination could be performed, the mutations affect both alleles, implying a complete loss of MLL2. Loss of MLL2 was confirmed at the protein level in DLBCL cell lines.

Conclusion: This highlights the benefits of both RNA-seq and whole genome sequencing (WGS) for generating a complete view of mutation in a cancer type. The enrichment of mutations in chromatin remodeling genes may suggest that epigenetic regulation plays an important role in the development and progression of lymphomas.

[558] Epigenetic Regulation of Amphiregulin and Eregulin and Their Impact on the Outcome of EGFR Targeted Therapeutics in Colorectal Cancer Cell Lines

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Introduction: Cetuximab, a monoclonal antibody against the EGF tyrosine kinase receptor (EGFR), has become a common therapeutic for patients harbouring different kinds of cancer. Yet, therapy response rates are not as high as predicted by conventional biomarkers (e.g. KRAS). Therefore, new predictive markers are urgently needed to better stratify patients prior to therapy. Candidates for such biomarkers comprise the genes for the EGFR ligands Amphiregulin (AREG) and Eregulin (EREG), since it was shown that AREG and EREG gene expression correlates with EGFR expression and may influence the cells’ sensitivity towards EGFR inhibition.

Material and Methods: Colorectal cancer cell lines with known mutation profiles were treated with Cetuximab and Gefitinib to determine their sensitivity or resistance profile. In addition, they were treated with the demethylating agent 5-aza-deoxycytidine (DAC) and Histone deacetylase inhibitors (HDACi) or transfected with an AREG expression plasmid. Afterwards their sensitivity towards EGFR-targeted therapeutics was determined by XTT measurement. CpG-methylation in the AREG and EREG regulatory regions was measured by 2nd generation sequencing.

Results and Discussion: DAC and several HDACi (Trichostatin A, Valproic acid) lead to an upregulation of AREG and EREG in a proportion of the cells tested. However, methylation analysis revealed no CpG methylation of the AREG promoter and CpG methylation of the EREG promoter only in RKO cells. While in untreated cells there was no correlation between AREG/EREG protein and mRNA levels and response towards EGFR targeted therapeutics, we observed an increase of sensitivity in some of the HDACi pre-treated cells and also following AREG transfection into Caco2 cells.

Conclusion: AREG and EREG expression is regulated in an indirect way by epigenetic mechanisms in colorectal cancer cells. Therefore therapeutic interference targeting epigenetic marks might influence the response to colorectal targeted therapies at the EGFR; however the mechanism remains to be determined.

[570] Prognostic Value of Kit and PDGFRα Mutations and Genetic Aberrations in Gastrointestinal Stromal Tumors

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Background: Activating mutations in Kit and PDGFRα tyrosine kinases are central to the pathogenesis of gastrointestinal stromal tumours (GISTs) and are associated with different clinical behaviour. Additional genetic aberrations were found in GISTs, demonstrating the involvement of other genes important in tumor progression. The aim of the study was to evaluate the prognostic relevance of different Kit mutations and genetic aberrations in GISTs.

Material and Methods: 240 GIST patients were examined for Kit (9, 13, 17 exons) and PDGFRα mutations (12, 14, 18 exons) and sequencing of DNA obtained from microdissected tumor sections. 46 tumor tissue DNA samples from GIST patients were screened for loss of heterozygosity (LOH) at 11 microsatellite loci on 1p, 9p, 14q, 15q and 22q.

Results: Kit mutations were found in 77.1% of GISTs, of them 64.6% were located in exon 11, 10.4% in exon 9. Mutations in Kit exons 13 or 17 were found in 5 GISTs. Mutations in PDGFRα were found in 11.2% of GISTs, of them 10.4% in exon 18 and 0.8% in exon 12. Typical substitution DB42V was found in 12 GISTs (5%). Wild type Kit and PDGFRα were found in 28 GISTs (11.7%), of them three cases with Carney triad, one gastric GIST with neurofibromatosis and four pediatric GISTs. Prognostic significance of such Kit mutations was evaluated. There was a trend of better survival for patients with PDGFRα mutation then with Kit mutation or wild type GIST. The higher overall survival prior to target therapy was shown for patients with duplication or point mutation in Kit exon 11 in comparison to exon 11 deletion. Allelic deletion in 11 microsatellite loci on 1p, 9p, 14q, 15q and 22q were found on 85% of GISTs, mostly, at 14q (59%) and 22q (50%). There was a correlation between different LOH pattern and survival in subgroups of GISTs. GISTs with PDGFRα mutation or Kit exon 11 point mutation had LOH at14q, while
GISTs withKITexon11deletionsrecoveredfrequentLOHalsoat22q,15p,and1p.GISTswithwildtypeKITandPDGFRAwerepullievedratherthanKITwithdifferentchromosomes.LOHN1pwasfoundexclusivelyinmetastaticandrecurrentGISTsin44%ofcases.SpecificgenelicofrequentLOHwereidentifiedon1p,15qand22qthatmaybecointegratedtoGISTspopulation.

**Conclusion:** The panel of eight CIMP markers was associated with GISTs prognosis.

**Introduction:** Aberrant methylation of DNA results in gene silencing and is frequently observed in tumour samples from patients with lung cancer. Methylation aberrations in tumors can also be detected in the draining lymph nodes, but it is unknown if the assessment of the nodal methylation status may identify more advanced stages of the disease.

**Objective:** To determine the relationship between the methylation status of five genes in metastatic lymph node and tumours samples obtained by endobronchial ultrasound with real-time guided transbronchial needle aspiration (EBUS-TBNA) and the presence of an advanced stage of lung cancer.

**Methods:** Nodal and tumour samples obtained with EBUS-TBNA and positive for lung cancer were flash-frozen with N2 and analysed. After the extraction of DNA with phenol-chloroform, DNA was chemically modified and the methylation status of DAPK, p16, RASSF1, APC and CDH-13 genes were determined by methylation-sensitive high resolution melting.

**Results:** The level of methylation in tumour cells obtained from lymph nodes and/or tumours accessible to EBUS-TBNA was higher in patients with more advanced stages of lung cancer, identified by a higher T staging.

**Conclusion:** There is a correlation between GIST survival and type of TK mutation and LOH of chromosomes. LOH on 9p was found exclusively in metastatic and recurrent GISTs and younger patients (p < 0.001; OR = 7.6). These three molecular markers were significantly associated to earlier stages.

**Copy Number Variation Analysis for Identification of Novel Disease-related Regions in Bladder Cancer**

**Introduction:** In order to perform a high-resolution analysis of CNV in bladder cancer, the Affymetrix GeneChip Human Mapping 250K Nsp chip platform was used. 49 tumor samples of bladder cancer patients at different clinical stages [17 (35%) clinical stage 1 (15 Ta and 2 Tis), 11 (23%) stage 2, 15 (31%) stage 3] and corresponding normal samples at different clinical stages [17 (35%) clinical stage 1 (15 Ta and 2 Tis), 11 (23%) stage 2, 15 (31%) stage 3] and corresponding normal samples (lymphocytes from the same patient) were screened for somatic CNV. We used Oligo (1.16.1), DNAcopy (1.28.0) and GenomicRanges (1.6.7) packages from the Bioconductor (2.9) project to perform our analysis. A threshold of >2.5 (amplification) and <-1.5 (deletion) concomitant at least two samples in the same tumor grade was used to identify potential CNVs. The DAVID Bioinformatics Resources 6.7 tool was used to perform the Gene Set Enrichment Analysis.

**Results:** According to our analysis, deletions (24%) are more frequent than amplifications (12%). As previously described in the literature, chromosome 9 short arm is the most frequent human genomic region affected by CNVs, being all deletions. We also detected genomic deletions located at 21q and 22q chromosome regions. Amplifications were detected at chromosome 7p, 13q and 17q. The analysis of enrichment Gene Ontology (GO) terms showed both amplified (p-value 0.0004) and deleted (p-value ≤ 0.0001) regions having the cell adhesion term overrepresented. If only amplified regions were analyzed, the following GO terms were overrepresented: cell motion (p-value 0.0006) and regulation of cell motility (p-value 0.0002).

**Conclusions:** In conclusion, our results show that the imbalance of deleted and amplified genes related to cell adhesion and motility may regulate tumor evolution and provide new insights to the understanding of bladder cancer biology.

**Methylation Profile in Oligodendrogial Tumours**

**Introduction:** Oligodendrogial tumours are primary brain tumours with a variable biological behaviour depending on the presence of 1p/19q deletion. Combined 1p and 19q loss occurs in a 50–90% of classic oligodendrogial tumours and a 30–50% of oligoastrocytomas. The presence of this deletion involves better prognosis and treatment response. It has been hypothesized the existence of 1p/19q tumour suppressor genes that are inactivated in these tumours. Recently, prognostically favorable CIMP+ (CpG
island methylation phenotype) tumours have been reported, pointing out the relevance of epigenetic mechanisms in gliomas. The major goal of this study was to analyze the methylation status of the whole genome in oligodendrogial tumours in order to obtain a methylation profile which would allow us to characterize these tumours and to identify candidate genes.

Material and Methods: We have included 35 oligodendrogliomas characterized for 1p/19q deletion (20 WHO grade II, 15 WHO grade III), 12 oligoastrocytomas (5 WHO grade II, 7 WHO grade III) and 5 normal brain samples. Methylation profiling analyses were performed by using Infinium HumanMethylation 450K BeadChip (Illumina®) which analyse 485,512 CpGs of the whole genome. We will validate our results by bisulfit sequencing and clonal bisulfite sequencing on selected candidate genes.

Results and Discussion: Overall, the tumoral and normal brain tissue analysis of the methylation profiles showed that methylation was predominantly distributed in the gene body and intergenic regions of the genome, while it was not concentrated in the promoter regions. The differentially methylated CpGs represent around 5% of the analysed CpGs. Among them, only 243 hypermethylated CpGs in oligodendrogial tumours were unmethylated in normal brain. These methylated CpGs were mainly located in islands of the promoter and gene body regions.

On another hand, we observed that tumours with combined 1p and 19q loss showed more frequent hypermethylation (52.7%) than tumours without deletion (44.7%). Hypermethylation in oligodendrogial tumours was distributed along all chromosomes and not preferentially located at 1p and 19q regions. Selected hypermethylated genes showing aberrant methylation in oligodendrogliomas were, among others, NUKA1, DLEU7, RTKN, KIAA0495, CFLAR and GDNF.

Conclusion: The whole genome methylation study allowed us to classify oligodendrogial tumours according to their methylation profiles which were related to the 1p/19q status. Higher levels of aberrant methylation (CIMP+) tumours were mostly identified in high-grade and 1p/19q deleted tumours. This methylation was not restricted to 1p/19q chromosomal regions. We propose candidate differentially methylated genes in oligodendrogial tumours.

**576 Detection of Genes and Pathways Involved in the Development of Aggressive Neuroblastoma Using Genome Copy Number Data**

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In this project, biopsies for the different subclasses of breast cancer (46 basal-like, 33 HER2+ / ICR- , 35 luminal A and 40 luminal B) were obtained from the Biological Center of Institut Curie and characterized by standard cytogenetics. Using Infinium HumanMethylation 450K beadchip (Illumina®) which analyse 485,512 CpGs of the whole genome. We will validate our results by bisulfit sequencing and clonal bisulfite sequencing on selected candidate genes.

Results: We present the results of separate analyses of each of these molecular levels, and we introduce methods that integrate information across multiple layers of data to pinpoint potential drivers of the disease. In particular, we propose an approach to identify cancer regions whose genetic alterations correlate with transcriptome-wide perturbation of gene expression. Such correlation suggests that these regions may harbor genes playing an important role in carcinogenesis. Using criteria based on co-expression and on prior biological knowledge, the method determines the genes in these regions that are most likely to be cancer drivers and may therefore be relevant as therapeutic targets. Using HER2+ tumours to illustrate the approach, we show that the method is indeed able to designate the HER2 oncogene as a likely driver.

Conclusions: With the addition of mRNA and protein data to these integrated studies, we hope that such approaches will suggest potential cancer driving genes as novel potential therapeutic targets.

**577 Modulation of Epigenetic States and Infant Immune System by Dietary Supplementation With (Omega-3) Polyunsaturated Fatty Acid During Pregnancy in an Intervention Study**

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Background: It is now recognized that the increase in human diseases like allergic disorders and cancer might be the result of a failure of normal immune regulation in early life. For example, early life exposure to tobacco and nicotine smoking have been identified to induce epigenetic changes in genes involved in allergy and asthma development.

Methods: We analyzed array CGH data for 270 NB patients present with segmental chromosome alterations. We explored the role of gene gains and losses in NB progression using local assessment of the differences in Kaplan–Meier curves with log-rank test (LRT) between groups of patients with different copy number status of each genomic region. We analyzed normalized LRT p-values (q-values) together with average copy number status (ACNS) of genomic regions. We selected genomic regions that corresponded to local extrema of the two curves: LRT q-value profile and ACNS profile along the genome. We discarded genomic regions for which the Cox analysis (including such additional parameters as age, gender and disease state) did not provide significant p-values. We added extra evidence to our predictions using available gene expression data for NB patients.

Results: We provide a list of genes, whose loss or gain has the highest predictive effect on the patient outcome. This list contains genes regulating chromatin during DNA repair, functioning in neurite outgrowth and axon guidance. Interestingly, the only important gene detected on chromosome 2 was MYCN. It is known, that mutations of another gene on chromosome 2, the tyrosine kinase receptor ALK, predispose to NB. As we expected, ALK did not come out from our analysis since we only studied whole gene amplifications and deletions.

Conclusion: We identified a list of genes (1) which are frequently lost or gained in NB and (2) whose gain or loss status has the highest association with a poor outcome in this region. Biological validation of these findings could provide insights into mechanisms of NB progression. In future, the use of a classifier based on our gene list may be a step forward in personalized medicine strategy for treatment of NB patients.
Detection of Hot Spot Mutations by Semi-conductor Sequencing of DNA Extracted From Formalin Fixed Paraffin Embedded (FFPE) Colon Rectal Cancer Samples

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The characterization of gene mutations in solid tumor samples is now a part of the clinical management of cancer patients in order to determine the best therapeutic option and it opens new avenue in the personalized medicine approach of cancer treatment. Various technical techniques have been developed in order to try to answer to the increase number of tests needed to be able to take a therapeutic decision and to deal with limitations: the poor quality of the DNA extracted most of the time from formalin fixed paraffin embedded (FFPE) tissues, the low quantity of DNA available, and the contamination of tumor sample by non-tumor cells. Alternative methods to the sanger sequencing are sensitive but limited in terms of number of mutation hotspots explored as well as the number of DNA quantity used and the high number of possible reads increasing the sensitivity of the method.

We tested 8 FFPE tumors samples derived from 6 lung cancers and 2 colon cancers. These tumors have been previously characterized for KRAS and EGFR mutation by TaqMan® Mutation Detection Assays based on Competitive Allele-Specific PCR technology (castPCR, LifeTechnologies). Five tumors were mutated for KRAS (2 colon and 3 lung cancers) and 3 were mutated for EGFR (3 lung cancers). The DNA was extracted from FFPE samples using Qiangen kit. The AmpliSeq™ Cancer Panel from Life Technologies was used allowing the amplification of different targeted region including those of KRAS and EGFR. A maximum of 10 nanograms of DNA were used as template for each tumor. Four tumors were sequenced using the Ion AmpliSeq™ Cancer Panel and the Ion 314 chip on the Ion TorrentTM PGM™ (1 tumor per chip) and 4 tumors were sequenced using a unique Ion 316 chip. In this last case we used barcodes to distinguish the different sample from each other. The sequences were analyzed using the Variant Caller plug-in of the PGM™ Torrent Server. The report was automatically generated including links to IGV software tool to view the sequences. The mean number of mapped reads per tumor was 399,005±44238, the average base coverage depth per tumor was 1809±357, the coverage at 100x was observed in 86.7±4.4% of the targeted sequences. All the predefined mutations in KRAS and EGFR were found. Additional mutations were found in APC, TP53, CTNNB1 and PIK3CA genes. Furthermore known polymorphisms were characterized in different genes. The coverage of targeted sequence appear to be enough to provide a sensitivity of 5%. The Ion AmpliSeq™ Cancer Panel tested seems to provide a cost effectiveness alternative to characterize lung and colon tumor samples in translational cancer research laboratory.

Parthenolide Inhibits Tumor Promotion – Epigenetic Regulation of P21

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Background: The promotion stage in the multistep process of tumorigenesis is NF-kB-dependent, epigenetically regulated, and reversible, thus, a suitable target for chemoprevention. We investigated whether the NF-kB inhibitor, parthenolide, currently in cancer clinical trials, attenuates tumor promotion by modulating the epigenetically regulated NF-kB target genes, p21 and cyclin D1, which play crucial roles in epidermal tumor promotion and carcinogenesis.

Material and Methods: Cell growth assays, soft agar colony formation, cell cycle analyses by flow cytometry, NF-kB gel shift and luciferase reporter assays, westerns, real-time PCR, chromatin immuno-precipitation (ChIP) assays, siRNA, tumor mouse models, tissue microarrays.

Results: Parthenolide selectively inhibited the growth of neoplastic keratinocytes while sparing normal cells using in vitro models of human and murine epidermal carcinogenesis. In JB6P+ cells, a model of tumor promotion, parthenolide attenuated promoter-induced cell proliferation and anchorage-independent growth, blocking promoted cells in S-G2/M phases. Furthermore, parthenolide decreased basal and promoter-induced NF-kB activity and modulated the expression of the NF-kB target genes, p21 and cyclin D1. In parthenolide-treated cells, p21 transcriptional regulation was achieved with high chromatin density at the cyclin D1 promoter. Using p21-siRNA and human colon carcinoma HCT-116 wild type and p21−/− clonal cell variants, we showed that p21 mediates cell sensitivity to parthenolide. Finally, parthenolide did not alter general histone deacetylase (HDAC) activity or global chromatin structure and synergistically inhibited the growth of epidermal tumor cells when combined with HDAC but not DNA methyltransferase inhibitors that are used in cancer clinical trials. In vivo, low parthenolide concentrations (0.25 mg/Kg) inhibited tumor growth of promoted JB6P+ cells in xenograft NMRI immuno-compromised mice, when administered intraperitoneally every other day over a ten-day period and stopped upon injection of tumors cells or stopped growth. Tissue microarray of mouse tumors showed that parthenolide decreased tumor cell proliferation, evaluated by Ki67, decreased p65/NF-kB levels, and increased p21 expression.

Conclusion: These results show that, at low doses, parthenolide inhibits tumor promotion and epigenetically regulates the expression of the NF-kB target gene, p21, which is crucial for parthenolide’s antitumor potential. Parthenolide is currently in cancer clinical trials and our result support its testing in epigenetic therapy whether alone or in combination with other drugs.

Identification of Functional Targets of Serous Ovarian Cancer


The development of more effective regimens is critical to achieve major advances in ovarian cancer therapeutics. In order to address the extensive heterogeneity exhibited by the disease of ovarian cancer, we have developed an expression-based classification scheme to further subdivide high-grade serous ovarian adenocarcinoma into five distinct subtypes (Epi-A, Epi-B, Mes, Stem-A and Stem-B) exhibiting more biological and clinical homogeneity. This classification scheme now provides us an opportunity to identify molecular targets specific for each of the multiple subtypes. Based on this scheme, we performed genome-wide functional screens to identify subtype-specific growth-promoting genes through the use of pooled siRNA lentivirus library. 88, 85 and 77 genes were found to be potentially important for the cell growth of Stem-A, Mes and Epi-A cell lines, respectively. Upon validation with individual siRNA, five genes were identified to be specifically relevant for cell growth in Stem-A subtype, which shows poor patient outcomes in clinics. Although additional studies are still required to provide insights on the mechanism, our findings demonstrated that there are indeed distinct patterns of growth determinants across the transcriptional subtypes, therefore implying a potential to develop subtype-specific regimens in the therapeutic of serious ovarian cancer.

CpG Methylation and Transcription Factor C-Myc Regulate Human Vav1 Expression in Hematopoietic and Lung Cancer Cell Lines

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Introduction: Vav1 is a signal transducer protein that functions as a guanine nucleotide exchange factor for the Rho/Rac GTPases in the hematopoietic system where it is exclusively expressed. Recently, Vav1 was shown to be involved in several human malignancies including neuroblastoma, lung cancer, and pancreatic ductal adenocarcinoma (PDAC). Although some factors that affect vav1 expression are known, neither the physiological nor pathological regulation of vav1 expression in non-hematological tissues is completely understood.

In the present study, we focused on understanding the mechanisms regulating the expression of vav1 gene transcription in hematopoietic and lung cancer cell lines. We demonstrated that mutations in putative transcription factor binding sites at the vav1 promoter affect its transcription in cells of different histological origin. Among these sites is a consensus site for C-Myc, a hematopoietic-specific transcription factor that is also found in Vav1-expressing lung cancer cell lines. Depletion of c-Myc using siRNA led to a dramatic reduction in vav1 expression in these cells. Consistent with this, co-transfection of c-Myc activated transcription of a vav1 promoter-luciferase reporter gene construct in lung cancer cells devoid of Vav1 expression. Together, these results indicate that c-Myc is involved in vav1 expression in lung cancer cells. We also explored the methylation status of the vav1 promoter. Bisulfite sequencing revealed that the vav1 promoter was completely unmethylated in human lymphocytes, but shows various degree of methylation in tissues that do not normally express vav1. The vav1 promoter does not contain CpG islands in proximity to the transcription start site; however, we demonstrated that CpG islands were related more with a single CpG dinucleotide at a consensus Sp1 binding site in the vav1 promoter interferes with protein binding in vitro.

Conclusion: Our data reveal two regulatory mechanisms for vav1 expression: binding of C-Myc and interruption of transcription factors binding by CpG
ALK Rearrangement and EGFR Copy Number Gains/mutations in Czech Non-Small Cell Lung Cancer Patients

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Introduction: The predictive roles of EGFR mutations and EML4-ALK rearrangement are the most clinically relevant findings in small cell lung cancer (NSCLC) patients. Gefitinib and erlotinib are available for the treatment of advanced EGFR-mutated NSCLC patients. Crizotinib, dual inhibitor of ALK and C-MET, was FDA approved for advanced NSCLC patients harboring EML4-ALK fusion. Incidence of ALK rearrangement in NSCLC cases is estimated at 2%-7% and diagnostics of this aberration have become very important.

Material and Methods: Prospective consecutive cohort of stage IIIb/IV NSCLC patients, diagnosed from February 2011 to January 2012, were screened for EGFR mutations using peptide nucleic acid (PNA) clamping. EGFR and ALK copy number gains were determined using FISH (positive in average 3 copies of gene/nucleus) and ALK rearrangement using ALK break-apart probe. We performed EML4-ALK translocation probe FISH with cut-off of >15% positive cells.

Results: EGFR and ALK alterations were examined in 185 NSCLC patients. All patients were Caucasians with median age 67 years (29-85) and representation of 63 women and 122 men. The most frequent subtypes were adenocarcinoma (47.8%) and squamous cell carcinoma (37.6%). FISH assay was inconclusive in 35 cases (18.9%) and EGFR mutation detection in 31 cases (23.8%). ALK and EGFR copy number, ALK rearrangement as well as mutational status of EGFR gene were determined in 130 NSCLC patients. No alteration was found in 83 cases (63.8%). EGFR copy number gain was found in 24 cases (18.5%), ALK copy number gain was observed in 21 cases (16.2%), simultaneous EGFR copy number gain was detected in 10 of them. EGFR activating mutations were found in 13 cases (10%). ALK rearrangement was found in 5 cases (3 women, 2 woman) by break-apart probe FISH. Rearrangement as well as fusion partner EML4 were confirmed by EML4-ALK translocation probe. Adenocarcinoma histology was confirmed in four cases, no histology subtype specification was described in last NSCLC case. ALK rearrangement was mutually exclusive in all cases, no concurrent EGFR mutations were found. In one case, ALK copy number gain was found simultaneously.

Conclusions: ALK rearrangement detection has become very important for NSCLC patients due to huge benefit from crizotinib therapy and should be part of routine diagnostic of NSCLC patients as ALK EGFR mutation detection. Clinical relevance of ALK copy number gain needs to be clarified.

Cooperating Tumor Suppressor Genes on Chromosome 8p Predict Survival Outcome in Hepatocellular Carcinoma

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Introduction: Most cancer genomes contain large heterozygous deletions of uncertain biological significance and large deletions of chromosome 8p commonly occur in epithelial tumors. Early studies on the RB and TP53 tumor suppressor genes suggested that such deletions can arise as one mechanism for loss of heterozygosity (LOH) and, consequently, it is often assumed they provide a ‘second hit’ event to inactivate a single tumor suppressor gene. TGs as genomic aberrations identified a definitive TSG within some cancer-associated deletions, e.g. chromosome 8p deletions, raising the possibility they occur through genomic instability or selection for the reduced activity of multiple genes. In another study, we performed expression data of human HCC patients was used to validate our findings in human cancer.

Results and Discussion: By targeting the mouse orthologs of genes frequently deleted on human 8p22 and adjacent regions, which are lost in approximately 50% of several other major epithelial cancers, we provide evidence that multiple genes on chromosome 8p inhibit tumorigenesis in mice and show that their co-suppression can synergistically promote cancer growth. Additionally, in human HCC patients, chromosome 8p genes are commonly co-deleted and the combined downregulation of the validated 8p tumor suppressors is significantly associated with poor survival in contrast to the downregulation of any individual gene reinforcing the functional role of those genes in the human disease.

Genomics of Metastatic Progression in Cutaneous Melanoma

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Introduction: Melanoma is one of the most aggressive human cancers. Patients with lymph node metastasis can show highly variable clinical outcomes, from several years disease free survival after excision of the primary tumor to extremely aggressive metastatic disease. In this study, we aim to compare the genomic and transcriptomic changes between lymph node and distant metastases in one poor prognosis case. We show that there are genetic changes in melanoma that suggest the survival of distant metastasis.

Material and Method: We have initiated this study with a poor prognosis patient, diagnosed with lymph node metastasis and showed the tumor distant metastasis to lung, 14.8 months after complete lymph node dissection. To reveal the genomic aberrations, we used a long-span pair-end-ditag (DNA-PET) sequencing approach to study copy number (CNVs) and structural variations (SVs) in lymph node and lung metastatic tumor samples. At the transcriptomic level, using paired-end RNA-seq (RNA-seq) sequencing, we comprehensively searched for gene expression level, alternative splicing, presence of fusion transcripts and single nucleotide variations. SOLID and illumina sequencing platforms were applied for DNA-PET and RNA-seq libraries, respectively. The sequenced reads were mapped back to the human reference genome (hg19). Normal and tumor genomes pair-comparison defined the cancer-specific profile. Integration of DNA-PET and RNA-seq validated data provided us with the comprehensive catalogue of somatic events in this patient.

Results and Discussion: Based on differential gene expression data, 313 genes are up-regulated in lung compare to the lymph node metastasis in this patient. On the other hand, CNV pair-wise comparison revealed 165 amplified genes in lung compare to the lymph node metastasis. Integration of these two gene lists provided us with the final list of 16 genes located on chr9 and chr12, amplified and over-expressed in lung compare to lymph node. The enrichment of the variety of SVs and occurrence of several translocations between chr9 and chr12 explains these amplifications which led to over-expression of genes on these chromosomes. We believe that there are gene cassettes on chr9 and chr12 which their specific amplification and over-expression function as driver cassettes for tumor recurrence. The correlation of these genes with the cancer phenotype will be addressed.

Conclusion: High-throughput sequencing technologies such as DNA-PET with increased sensitivity for rearrangement detection are determined to provide comprehensive patterns of genomic aberrations underlying tumor progression and maintenance.

Comparison of DNA Methylation Markers in Advanced Stage, High Risk Neuroblastoma Patients

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Introduction: Most of the high risk, stage 4 Neuroblastoma (NB) patients experience a poor outcome despite novel multimodal therapeutic protocols, but the limited number of patients available has severely restricted the scope of this clinical trial. Methylation of the Protocadherin B (PCHD8) gene was analyzed in a cohort of patients ranging from Stage 1 to Stage 4 seemed to be a really promising biomarker in a Neuroblastoma. We analyzed this biomarker specifically in stage 4 patients at high risk.

Material and Method:We developed a pyrosequencing assay to measure the methylation level of 17 genes of the PCHD8 cluster and we analyzed 106 tumors of high risk stage 4 NB patients comparing the results with stage 4 at low risk and stage 1 at very low risk NB patients.

methylitation in 5’ regulatory sequences. Mutation of other putative transcription factor binding sites suggests that additional factors regulate vav1 expression as well.

582 ALK Rearrangement and EGFR Copy Number Gains/mutations in Czech Non-Small Cell Lung Cancer Patients

584 Cooperating Tumor Suppressor Genes on Chromosome 8p Predict Survival Outcome in Hepatocellular Carcinoma

585 Genomics of Metastatic Progression in Cutaneous Melanoma

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We assessed a multivariate analysis considering all clinically important parameters in stage 4, high risk NB patients such as MYCN amplification and including also the methylation of Stratifin (SFN) gene a biomarker statistically related to survival in this group of patients. **Results and Discussion:** DNA methylation of PCDHB cluster is lower in stage 1 compared to stage 4 patients, but in stage 4 high risk patients its predictive power is absorbed by other clinically relevant parameters while methylation of SFN gene appears as an independent predictor of outcome which identifies high-risk patients surviving more than 60 months with methylation levels comparable to tumors deriving from lower risk patients. A particular threshold of methylation of SFN gene seems to be a better predictor of prognosis discriminating the survival in high-risk patients. This could indicate that higher level of methylation of SFN gene could be associated to a greater aggressiveness of the disease. Furthermore there is a higher level of methylation of PCDHB cluster in a group of stage 4 high risk NB patients with bad prognosis respect to patients with good outcome in stage 1 underlining the possible relation between outcome and DNA methylation but also, as a proof of principle, between INSS stadiation and DNA methylation of specific targets. **Conclusion:** Methylation level of PCDHB cluster doesn’t act as a surviving biomarkers in high risk stage 4 NB patients and we hypothesized that a subset of patients considered at high risk – but displaying low levels of SFN methylation – could be assigned at a lower risk group.

**DNA Methylation Profiling Identifies Luminal A Breast Tumors With Poor Survival**

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**Background:** Based on gene expression profiles of breast cancer tumors five subgroups have been identified (Luminal A, Luminal B, ERBB2 enriched, basal-like and normal-like) and the group showing different survival. Luminal A is usually the largest group and has the best prognosis. Lately, analyses have identified three groups based on DNA methylation (Cluster 1−3) that also show different survival. The concordance between gene expression groups and DNA methylation groups is strong, though interestingly, the Luminal A tumors are split quite evenly between the cluster with best survival and the cluster with intermediate survival. Based on the split between Cluster 1 and 3, patients having Luminal A tumors show different survival. This study set out to further investigate the two groups of Luminal A tumors.

**Materials and Methods:** DNA material from 80 breast tumors were analyzed by Illumina GoldenGate interrogating 1505 CpGs, and the findings have been validated in a dataset of the same platform. All 93 breast tumors were analyzed on another platform (Illumina Infinium 27k methylation array) interrogating more than 27 thousand CpGs. Whole genome expression profile was available for all samples.

**Results:** Within the luminal A tumors, 41 genes were found differentially methylated between Cluster 1 and Cluster 3 (FDR <5%), and these included BIRC4, CD40, CDKN1C, EGFR, ESRR2, ICAM1, KIT, MAS1, SFRP1, TERT, WNT1, and WNT1. Further, this gene list was used to perform hierarchical clustering on Luminal A breast tumors analyzed by Illumina 27k methylation array, and also these tumors were split in two groups. When applying Kaplan−Meier survival analysis on these two groups, a significant difference was observed. Further, the patients were divided in two groups: survivors and non-survivors, and nine of the 41 genes were found with significant differences in methylation level between survivors and non-survivors.

**Conclusions:** Earlier work has shown that Luminal A tumors are split between two DNA methylation clusters and that these show different survival. Here we show which genes drive the separation of the Luminal A tumors into two groups by DNA methylation, and we show that the difference in survival is apparent on multiple dataset and analyzed on different platforms.

**Exome Sequencing of Adenoid Cystic Carcinoma**


**Introduction:** Adenoid cystic carcinoma (ACC) is a rare malignancy of the salivary gland. Although slow growing, it has a progressive and heterogeneous nature with poor long term prognosis. The primary treatment is surgical resection followed by radiotherapy. Nevertheless, the majority of patients succumb to metastatic disease, with very limited therapeutic options. To date the most notable disc gene in the genetics of ACC has been the identification of recurrent translocations of t(6;9)(q22−23;p23−24) resulting in the fusion of MYB with NFIB. However, very little is known about the contribution of other genes to this disease.

**Material and Method:** The coding exons of 21,416 protein coding genes and 1664 microRNAs were sequenced in twenty four primary ACC and matched normal salivary gland parenchymal samples. The set consisted of roughly equal numbers of cribriform and the more aggressive solid histology tumours, which are associated with poorer prognosis. Solution phase capture was performed using the Agilent SureSelect Human All Exon 50Mb kit and each exome was sequenced using a 76bp paired-end protocol on an Illumina GAII DNA Analyser to a minimum depth of 30X across 70% of the target exons. Putative somatic mutations were confirmed using capillary sequencing of both tumour and normal DNA. In addition, copy number changes were investigated using Affymetrix SNP6 microarrays analysed using ASCAT.

**Results and Discussion:** In total over 300 somatic mutations were identified, with an average of 13 mutations per sample. Copy number analysis confirmed previous observations of recurrent losses of 1p36, 6q, 9p and 12q. A subset of the mutations were classified as driver mutations. These mutations are predisposed to confer a selective advantage on cancer cells and have previously been causally implicated in cancer. Potential driver mutations were identified in 17 known recessively acting cancer genes and dominant oncogenes. In combination with additional mutations in a number of genes not previously implicated in cancer, these mutations provide evidence for the involvement of a number of pathways in ACC biology.

**Conclusion:** This systematic analysis of exome sequences in ACC extends the knowledge of the genes and pathways involved in this tumour type and provides an insight into potential therapeutic targets for this disease.
The DNA Methylation Landscape of Paediatric Acute Lymphoblastic Leukemia

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Introduction: Acute lymphoblastic leukaemia (ALL) is the most common childhood malignancy, accounting for 25% of all childhood malignancies in the Nordic countries. DNA methylation plays an important role as a modulator of gene expression and in the control of cell differentiation, thus DNA methylation could be one of the factors that enhance the malignant transformation of precursor blood cells into leukemic cells. DNA methylation may also contribute to the variation between ALL patients in clinical outcome and response to treatment.

Materials and Methods: Using Infinium Methylation BeadChips, we determined the methylation levels of >450,000 CpG sites in 1,002 samples, including 850 bone marrow samples collected at the time of ALL diagnosis from patients in the Nordic countries. Follow-up bone marrow samples from ALL patients taken during remission and purified CD19+, CD3+, and CD34+ cells isolated from healthy blood donors were included as controls. Four representative ALL patients were subjected to whole-genome bisulfite sequencing (BS-seq) at high coverage.

Results and Discussion: The DNA methylation levels (β-values) determined with the BeadChips were highly correlated to those measured by BS-seq of the same individuals (Pearson’s R >0.95). The ALL samples displayed higher variability and a distinct overall increase in methylation levels compared to healthy blood cell populations. Over 25k of the 450k CpG sites were differentially methylated in ALL cells with a median absolute difference in methylation levels >30% compared to the controls. Of these, 79% were hypermethylated in the ALL cells and predominantly located in CpG islands and nearby the transcription start site. In contrast, the majority of the hypomethylated sites were located outside of CpG islands, in particular in CpG sites flanking islands and in gene bodies. Currently, multivariate analysis of variation in DNA methylation levels across the genome against clinical outcome is underway.

Conclusion: Preliminary results show that the array-based assay and BS-seq is highly reproducible. Several methylation changes that will serve as candidates for further investigation into the molecular role of DNA methylation in ALL etiology and clinical outcome of ALL patients have been identified. This study has been approved by the Nordic Society for Paediatric Hematology and Oncology.

Lack of MicroRNA-101 Can Cause E-cadherin Functional Deregression Through EZH2 Overexpression in Intestinal Gastric Cancer

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Introduction: E-cadherin expression disruption is commonly observed in metastatic epithelial cancers and a crucial step in gastric cancer (GC) initiation and progression. As aberrant expression of microRNAs often perturb the expression of pivotal cancer-related genes, we characterised and dissected a pathway that causes E-cadherin dysfunction via loss of microRNA-101 and upregulation of EZH2 expression in GC.

Material and Methods: MicroRNA microarray expression profiling and Array-CGH were used to confirm miR-101 involvement in GC and validated with quantitative real-time PCR and quantitative Snapshot genomic PCR. EZH2 and E-cadherin mRNA and protein levels were determined by real-time PCR and western-blot/co-immunofluorescence. CDH1 mutation, methylation and LOH were determined. Gain/loss of function experiments were done in KatoII cells. E-cadherin functionality was assessed by immunofluorescence and flow cytometry.

Results: We confirmed that mir-101 was significantly downregulated in GC (P <0.0001) in comparison with normal gastric mucosae, and at least in 65% of the GC cases analysed, this downregulation was caused by deletions and/or microdeletions at miR-101 genomic loci. Moreover, around 40% of cases showing mir-101 downregulation, displayed concomitant EZH2 overexpression (at the RNA and protein levels) which, in turn, associated with loss/absent E-cadherin expression. Interestingly, this occurred preferentially in intestinal type GCs retaining allele(s) untargeted by classical CDH1 inactivating mechanisms. We also demonstrated that mir-101 gain of function or direct inhibition of EZH2 in Kato III GC cells, led to a strong depletion of endogenous EZH2 and consequent rescue of E-cadherin membranous localization, mimicking results obtained in clinical GC samples.

Conclusion: In conclusion, we show for the first time that deletions and/or microdeletions at both miR-101 genomic loci cause mature miR-101 downregulation and subsequent EZH2 overexpression and E-cadherin dysfunction specifically in intestinal-type GC.

Are Epigenetic Mechanisms Responsible for Silencing the Calcium Sensing Receptor in Colorectal Cancer?

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Introduction: Epidemiological studies suggest a role for calcium in prevention of colorectal cancer (CRC). The calcium sensing receptor (CaSR) probably mediates the antiproliferative action of calcium in colon. The CaSR expression decreases during tumor progression in human CRC. We hypothesized that epigenetic mechanisms like DNA hypermethylation and histone deacetylation might be responsible for silencing the expression of the calcium sensing receptor in colorectal tumors.

Material and Methods: We analyzed CaSR mRNA and protein expression in CRC tumors and cell lines by real time qRT-PCR and immunofluorescence. Bisulfite sequencing was used to determine the methylation pattern of two regions in the second promoter of the CaSR. We treated colon tumor cell lines with 5-aza-2-deoxycytidine (5-aza-dc), a DNA methyltransferase inhibitor, and/or Trichostatin A (TSA), a histone deacetylase inhibitor to induce the expression of the CaSR.

Results and Discussion: In CRC patients we observed a significant downregulation of CaSR mRNA expression (P <0.0001) in tumor tissues compared with the respective adjacent mucosa from the same patient. Immunofluorescence staining confirmed downregulation of the CaSR protein in tumors also. Bisulfite sequencing of CaSR in CRC cell lines showed dense methylation of the second region. Although the methylation ranged from 2–70% among patients, there was no difference in the methylation pattern between tumor and the respective adjacent mucosa in any of the analyzed regions. Treatment with 5-aza-dc and TSA caused only modest increase of the CaSR expression, despite the presence of densely methylated CpG islands in the promoter of the CaSR.

Conclusion: In our patient cohort the loss of CaSR expression in colon cancer is independent of DNA hypermethylation and histone deacetylation.

Modeling Prostate Cancer Oncogenesis Through Developmental Alterations in Zebrafish

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Introduction: Whole Exome and Genome sequencing has nominated the Spalt-like POZ protein (SPOP) as the most frequently mutated gene in localized prostate cancer at a frequency of up to 14% (Barbieri et al. in revision). SPOP functions as a substrate recognition subunit of a Cullin3-containing E3-ubiquitin ligase and is highly conserved among vertebrates. Although zebrafish do not have prostate glands, we explored the characterization of pathways altered as a consequence of the most frequent SPOP mutation, Zebrafish is a genetic model organism, which permits functional characterization of oncogenes and developmental pathways. The high level of conservation between zebrafish and human and the relative ease of genetic modification make zebrafish an excellent vertebrate model organism to characterize mutations found in next generation sequencing studies of human cancers.

Material and Methods: Wildtype AB/Tuebingen and p53<sup>M214K</sup> animals, a p53 point mutant in the DNA-binding domain were used for this study. We designed splice blocking and translation inhibiting morpholinos against a Cullin3-containing E3-ubiquitin ligase and is highly conserved among vertebrates. Although zebrafish do not have prostate glands, we explored the characterization of pathways altered as a consequence of the most frequent SPOP mutation, Zebrafish is a genetic model organism, which permits functional characterization of oncogenes and developmental pathways. The high level of conservation between zebrafish and human and the relative ease of genetic modification make zebrafish an excellent vertebrate model organism to characterize mutations found in next generation sequencing studies of human cancers.

Results and Discussion: An initial analysis of SPOP protein sequence revealed a high degree of conservation between zebrafish and human (98.9% amino acid identity). We performed morpholinobased knockdown of SPOP, which led to a highly reproducible phenotype characterized by malformations of brain development and overall body plan. Microinjection of human SPOP mRNA rescued this phenotype confirming the specificity of the morpholinobased knockdown. We performed RNA-sequence on zebrafish embryos after SPOP morpholin injection in order to identify pathways regulated by SPOP in these studies. SPOP regulation and activation of the DNA-damage response pathway. Furthermore, we overexpressed the most common SPOP mutant F133V in order to investigate the effect of this mutation on SPOP function. This analysis resulted in a...
significantly more efficient rescue of SPOP knockdown compared to wildtype SPOP, suggesting that F133V is a gain of function mutation.

Conclusion: In this study, we describe the use of zebrafish to evaluate the function of Spop in development. Our studies demonstrate that Spop is required for early organogenesis of the CNS. We identified Spop to be a key regulator of developmental processes potentially by controlling cell cycle and DNA-damage responses. This study emphasizes the use of zebrafish as an emerging in vivo model to functionally characterize genetic alterations identified in large-scale next generation DNA-sequencing studies. We are currently analyzing transcriptome data from human prostate cancer tissue and cell lines to verify the relevance of the molecular alterations identified by Spop knockdown in zebrafish for human cancer.

[596] Genetic and Epigenetic Alterations in the Embryonal Tumor Hepatoblastoma

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Embryonal tumors are rare malignancies originating from primary cells that have acquired somatic mutations. Hepatoblastoma (HB) is the most common embryonal tumor in children and adolescents, consisting of a solid embryonal tumor that presents high mortality and rapid progression. Molecular data on HB are still scarce and remain inconclusive. In the present study we were particularly interested in the delineation of somatic copy number alteration (SCNAs) and genome-wide DNA methylation patterns. We have investigated 5 HB samples. ArrayCGH with a 180K oligoarray platform (OGT) was employed to detect the SCNAs, using for analysis the Nexus 6 software (Biodiscovery). The global profile of genomic imbalances showed only a few alterations in each sample (average number of SCNA per tumor = 2.8), which indicates that HBs have less genomic instability than most solid tumors. Four out of five tumors exhibited high gain of a small segment in a region previously reported as altered at chromosome 3p. Initially, we detected a small amplified region at 2q, narrowing the minimum chromosome segment recurrently amplified. Two HB harbored a high level gain at 5q, encompassing among others genes a mRNA. Gains in mosacite w were found at 1q, 3p and X, in addition to loss of a segment at 22q including a known cancer susceptibility gene yet not related to HB.

Evaluation of the global DNA methylation pattern was performed with the 450K BeadArray platform and analyzed with the Genome Studio software (both from Illumina). A preliminary analysis disclosed 74 CpG sites significantly more methylated in tumors than in normal liver, nearly half of which lying in genes involved in metabolic processes. In complementary, we detected 34 de-methylated CpGs in HB samples, affecting genes involved mainly in cell fate commitment and epithelium development.

The group of HBs investigated will be expanded to contribute to elucidation in some available external microarray datasets, where it showed very good biased genomewide approach.

[597] Using an in Vitro Model of Epithelial-Mesenchymal-Epithelial Transitions to Uncover Novel Biological Mechanisms

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Background: Epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) are fundamental mechanisms controlling multiple events during embryonic development and cancer. Cancerous cells undergoing EMT, exhibit a mesenchymal-like phenotype with concomitant polarity loss, increased invasibility and apoptosis resistance, features predicted to enable metastasis. The establishment of cancerous cells at novel locations is a key event in metastasis and only possible due to expression plasticity characteristic of cells that are able to undergo MET. Many molecules have been proved to induce EMT and others to be modulated by EMT. However, never was the variation of whole transcriptome been assessed during these crucial biological processes. Therefore our aim was to assess the whole transcriptome variations in an in vitro model of EMT and MET, in order to clarify major biological changes underlying these processes.

Methods: We have reproduced EMT and MET in vitro by treating a normal mouse mammary cell line (EpH4) with/without TGF-$\beta$1. DNAseq treated total RNA extracted at distinct EMT/MET-timepoints was then subjected to whole transcriptome sequencing (Illumina GA). Bioinformatic analysis was performed using in house pipelines and commercially available software (Ingenuity Systems Pathway Analysis Software and Database for Annotation, Visualization and Integrated Discovery Software). RNA expression alterations were verified by qRT-PCR, protein expression by Western Blot, immunoprecipitation and immunofluorescence and DNA methylation alterations by bisulfite treatment. Metabolism intermediates were measured using ELISA.

Results: We confirmed the occurrence of EMT and MET via analysis of the differential transcription of classical epithelial and mesenchymal markers. In our in vitro EMT/MET model, we have found several thousands of genes differentially expressed and our bioinformatics analysis correlated this differential activation with uncounted cancer and metastasis related pathways. Moreover, we have uncovered novel biological mechanisms underlying EMT/MET such as differential glycosylation of E-cadherin, alternative activation of metabolic pathways and novel epigenetic mechanisms, underlying activation/repression of recently annotated genes.

Conclusions: We were able to establish a dynamic in vitro model of EMT/MET, which has enable us to uncover novel biological mechanisms using a non-biased genome wide approach.

[598] Follicular Thyroid Cancer Molecular Markers Validation in FFPE Material

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Introduction: Follicular thyroid cancer (FTC) is diagnostically challenging as its histological morphology is similar to a follicular thyroid adenoma (FTA). In the last decade many gene expression studies were performed to improve a relatively high misclassification rate in routine histopathological diagnosis. One of the most interesting studies was Borup et al. 2010 that is the largest follicular thyroid tumour database published so far.

Materials and Methods: Gene expression profile was carried out in 52 samples (25 follicular carcinomas and 27 adenomas) by HG-U133 Plus 2.0 oligonucleotide microarrays (Affymetrix). Whole dataset was analysed by Singular Value Decomposition to identify the major sources of variability. In the next step, a subset of our well-defined samples (cordinates of diagnosis between two experts in thyroid pathology) and samples from Borup et al. were combined in one powerful supervised analysis to look for the most important differentially expressed genes. Subsequently 8 transcripts, that came as a result of the analysis were validated by quantitative PCR using formalin-fixed paraffin-embedded (FFPE) material.

Results and Discussion: Immunologic response was the strongest supersgene discriminating FTC and FTA in unsupervised analysis (it accounted for 14.5% of the total variance). The second important source of variability was the proliferation rate of thyroid cells.

5 of 6 genes selected from supervised analysis (ELMO1, EMCN, ITIH5, KCNA1, SLC20A1) could be amplified by quantitative real-time PCR in FFPE material in the independent set of samples (N = 71). Validated genes were all significantly (p < 0.05) down-regulated in FTC when compared to FTA. The 3-gene classifier build on genes described above had a sensitivity of 71% and specificity 72%, when tested on QPCR dataset. The classifier was also tested in some available external microarray datasets, where it showed very good performance.

Conclusion: ELMO1, EMCN, ITIH5, KCNA1 and SLC20A1 are genes important for follicular thyroid malignancy development and information about their level can be helpful in FTC diagnosis. This work was supported by Ministry of Science and Higher Education grant nr N401 072837 and N403 194340 and by Foundation of Polish Science International PhD Projects (MPD) program.

[599] Up-regulation of ERL1, p53 and p21 Sensitizes Tamoxifen Resistant Breast Cancer Cells to Tamoxifen

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Breast tumors expressing estrogen receptors (ER $\alpha$ and $\beta$) respond well to therapeutic and preventative strategies with Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen. However, a number of breast cancers are either resistant or develop resistance to tamoxifen and other SERMs. The promoters of ER$\alpha$ and ER$\beta$ are rich in CpG islands making them susceptible to epigenetic modifications. Methylated CpG clusters are known to
be associated with gene silencing. Our hypothesis is that tamoxifen resistant breast cancer cells can be sensitized to tamoxifen after treatment with either a demethylating agent (such as 5-AZA) or a histone deacetylase inhibitor (such as trichostatin, TSA) or their combination. These treatments are expected to activate the transcription of ER-α and ER-β m-RNA and produce functional protein reversing in this manner tamoxifen resistance. Using the RTT assay we determined the growth rates of MCF-7, MCF-7/TAM-R and MDA-MB-231 in the presence of 5-AZA, TSA and their combinations in the presence or absence of 4-OH tamoxifen. We identified the conditions (i.e order of inhibitor addition and concentration) that reverse tamoxifen resistance and those providing the maximum cell death of the MCF-7 and MCF-7/TAM-R. We found that pretreatment with 5-AZA (2 μM) or TSA (0.1 μM) enabled tamoxifen (10 μM) to produce a considerable cell death (over 50%). Interestingly, MCF-7/TAM-R cells that normally do not express ER-α, after 24 hours of treatment with TSA and 5-AZA produced a marked increase in the expression of ER-α as determined by both Western Blot and fluorescence microscopy. Following 48 hours of treatment the levels of ER-α were further increased. Additionally, the combination of 5-AZA and TSA increased the levels of p21 by 10-fold compared to TSA alone and it was not further increased by the addition of 4-OH tamoxifen. Moreover, following 48 hours treatment with 5-AZA/TSA and tamoxifen the levels of p21 and p53 were substantially increased compared with the vehicle control. These results show: (a) the reversal of tamoxifen resistance can be achieved with relatively low (non-toxic) concentrations of 5-AZA/TSA, and (b) the upregulation of ER-α, p53 and p21 are essential components of the molecular pathway leading to this reversal. These findings may find applications in clinical therapeutic protocols in the treatment of tamoxifen resistant breast cancer.

[600] Agreement Between SNP6.0 and Cytogenetic 2.7M Whole-genome Microarrays From Affymetrix in DLBCL. J. Bodker1, P. Johansen2, C. Gyrup3, A. Schmitz1, P. Jensen4, H. Johnsen5, M. Bagstedt1, K. Dybkær1, M. Nyegaard1.

The high degree of consistency between the array types may play a pivotal role in the cancerogenesis of breast cancer. Studies are in progress to determine whether KEAP1 abnormalities may contribute to disease progression prediction and response to therapy in these patients.

[029] Frequent Epigenetic inactivation of KEAP1 Gene in Breast Cancer. R. Barbaro1, B. Pasquini1, C. L. Muscarella1, A. La Torre1, D. Trombetta1, R. Murgia1, VM. Falco1, E. Maiello1, P. Paerella1, J. I. RCCS Casa Solleva Della Soferza, Oncology Laboratory, San Giovanni Rotondo(FG), Italy, 1RCCS Casa Solleva Della Soferza, Breast Unit, San Giovanni Rotondo(FG), Italy, 1RCCS Casa Solleva Della Soferza, Department of Oncology, San Giovanni Rotondo(FG), Italy, 1RCCS Casa Solleva Della Soferza, Laboratory of Oncology, San Giovanni Rotondo(FG), Italy.

Background: The Nf2/Keap1 pathway is a master regulator of several redox-sensitive genes implicated in resistance of tumour cells against chemotherapeutic drugs. Recent data suggest that epigenetic mechanisms may play a pivotal role in the regulation of KEAP1 expression.

Material and Methods: We determined KEAP1 promoter methylation status in 50 breast cancers (BC), and 6 normal breast tissues (NBT) obtained from breast cancer patients. Using pyrosequencing and quantitative methylation-specific PCR (qMSP) we determined the KEAP1 promoter methylation status in 50 breast cancers (BC), and 6 normal breast tissues (NBT).

Results: We determined KEAP1 promoter methylation status in 50 breast cancers (BC), and 6 normal breast tissues (NBT). 5-AZA/TSA produced a marked increased in the expression of ER-α as determined by both Western Blot and fluorescence microscopy. Following 48 hours of treatment the levels of ER-α were further increased. Additionally, the combination of 5-AZA and TSA increased the levels of p21 by 10-fold compared to TSA alone and it was not further increased by the addition of 4-OH tamoxifen. Moreover, following 48 hours treatment with 5-AZA/TSA and tamoxifen the levels of p21 and p53 were substantially increased compared with the vehicle control. These results show: (a) the reversal of tamoxifen resistance can be achieved with relatively low (non-toxic) concentrations of 5-AZA/TSA, and (b) the upregulation of ER-α, p53 and p21 are essential components of the molecular pathway leading to this reversal. These findings may find applications in clinical therapeutic protocols in the treatment of tamoxifen resistant breast cancer.


Background: Thymine DNA glycosylase (TDG) belongs to the superfamily of the uracil DNA glycosylases and is the first enzyme in the base excision repair pathway that removes thymine from the G-T mismatches at CpG sites. Besides its antimutagenic role in DNA repair, the versatile TDG plays a crucial role in maintaining appropriate epigenetic regulation by interacting with transcription factors and histone acetylases, and by acting in the demethylation process of genes previously silenced by promoter methylation and hence reactivating them. Therefore the mechanisms by which proper functioning of TDG is assured may be critical for genetic and epigenetic stability. The tumor suppressor TP53 has essential roles in maintaining genomic stability: p53 exerts its control over several DNA-repair pathways, either directly or indirectly. However, so far, there is no information on whether p53 may control TDG.

Materials and Methods: Different cell lines mutated in TP53 (MN1 and MDD2 - isogenic breast cancer cell lines harboring either wild-type or inactivating p53, respectively) and MDA-MB-231 - isogenic breast cancer cell line harboring a temperature-sensitive p53, were used.

Results and Discussion: In this study we show that wild-type p53 transcriptionally regulates TDG expression. ChIP and lucerase assays revealed that wild-type p53 binds to a TDG promoter domain containing two p53 consensus response elements (p53RE), inducing its transcription. Using a panel of cell lines we demonstrated that TDG mRNA and protein expression levels are induced by DNA damage in a p53-dependent manner. Although not statistically significant, methylation levels of the analysed gene promoters were found slightly decrease in Li-Fraumeni p53 mutation carriers subjects,
suggesting that TP53 mutational status may affect the methylation process possibly through modulation of TDG activity.

Conclusions: Together these results show that TDG expression is transcriptionally regulated by p53, suggesting that loss of p53 function may affect the wide range of essential functions controlled by TDG, thus leading to cellular genetic and epigenetic instability. 

Cancer Genomics, Epigenetics and Genomic Instability. Mutational Processes Shaping the Genomes of Twenty-one Breast Cancers


The Wellcome Trust Sanger Institute, Cambridgeshire, United Kingdom

All cancers carry somatic mutations. The set of somatic mutations observed in a cancer genome is the aggregate outcome of the activity of one or more biological processes that have been operative throughout the lifetime of a patient. Each of these biological processes can be characterised by the pattern of mutations that it leaves on the cancer genome. The pattern of mutations or mutational signature characterising each process will be determined both by the underlying mechanisms of DNA damage and of DNA repair that constitute the biological process. The final catalogue of somatic mutations observed in a cancer genome will thus be determined by the strength and duration of exposure to each of the biological processes that have been operative in that cancer. We set out to extract the mutational signatures characterising the biological processes that have been operative in the 21 breast cancers studied. We generated catalogues of somatic mutation of all classes of mutation from twenty-one whole-genome sequenced breast cancers using an integrated suite of bioinformatic algorithms. Mathematical methods were applied in order to extract features of the underlying mutational signatures. Multiple distinct substitution signatures and their relative contribution to each cancer genome, were discernible. In addition, other distinctive phenomena have been unearthed by analyses of breast cancer genomes at this scale. This study harnesses the full scale of whole-genome sequencing technology providing insights into hitherto unrecongnised mutational signatures present in breast cancer genomes.

Analysis of Allelic Loss in BRCA2999del5 Mutated Breast Tumors

S.T. Reyndisdottrí, O.A. Stefansson, H. Bjarnasson, H. Hilmarsdottir.

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Background: Inherited heterozygous mutations of the tumor suppressor gene BRCA2 predispose carriers to breast cancer and other cancer types. Somatic deletion of the wild-type allele has been considered essential for neoplastic transformation. Recently, it was shown in a murine model that a familial pancreatic cancer that retention of the wild-type BRCA2 allele does occur in pancreatic adenocarcinoma tumor cells. Furthermore, our group has reported the retention of the wild-type BRCA2 allele in a subset of BRCA2 mutated breast tumors from women carrying the inherited BRCA2 999del5 mutation. Loss of the wild-type allele in these breast tumors was found to be associated with bad prognostic markers in tumors displaying luminal phenotypes. Here, we look at BRCA2 wild-type allele loss in a larger group of BRCA2 999del5 mutated breast tumors to further define the frequency and relevance of BRCA2 wild-type allele loss in relation to breast cancer, in BRCA2 999del5 mutation carriers.

Materials and Methods: Tumor DNA from 59 breast tumors derived from BRCA 999del5 mutation carriers was examined for allelic loss. BRCA2 allele specific quantitative PCR (7500 Realtime PCR System; Applied Biosystems) was performed using the TaqMan method with a single BRCA2-specific MGB-probe 5’-end-labeled with FAM, a single BRCA2-specific forward primer, and two allele-specific reverse primers. The BRCA2 wild-type- to mutant allele ratios were quantified by measuring differences in fluorescence intensity of FAM performed in duplicate, and the Ct values were averaged. The percentage of wild-type allele loss was calculated by the following equation: (2−Ct mutation/Ct wild) × 100, where Ct = (Ct of allele 1 − Ct of allele 2).

Results: The percentage of wild-type allele loss in breast tumors from BRCA2 999del5 mutation carriers ranged from 7-93%, 15 of 59 (25.4%) breast tumors did not show loss of the BRCA2 wild-type allele. Using the criteria that wild-type allele loss is present when the percentage of loss is 30% or more. These data will be further analysed with respect to clinical and prognostic factors, such as tumor phenotypes (luminal A or B, triple negative or basal-like), tumor staging and survival. Studies of wild-type allele loss in BRCA2 999del5 tumors might be an important tool for identification of patients that may benefit from treatment with poly-ADP-ribose (PARP1) inhibitors.

Conclusions: These findings indicate that loss of the BRCA2 wild-type allele is not always a required event for carcinogenesis in BRCA2 999del5 mutation carriers.

Defining the Molecular States of Cancer and Stem Cells Through Integrative Transcriptional and Genomic Analysis


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Introduction: Despite intensive studies over the last decade, molecular complexity of cancer still remains as the limiting factor for developing efficient therapies against the disease. However, scientists succeeded to determine common alterations between most types of human cancer. Each of these alterations is the result of defeating an anti-cancer regulation mechanism that maintains normal cell proliferation by various signaling pathways. Some of these pathways are normally involved in developmental process by maintaining the self-renewal ability of stem cells. Self-renewal is tightly regulated by the internal and external mechanisms. Unlike stem cells, cancers cells mimic the self-renewal, but lack this tight control. We hypothesize that the stemness regulatory ‘cassette’ that serves limitless replicative potential and unregulated state is a measure of cancer virulence. We believe that this cassette is established through different molecular and genetic mechanisms in each cell type, so it can be separated from invasion and instability in a precise and defined manner.

Method: To test our hypotheses, we set up a system reconstruction model consisting of iPS and transformed cells as stem and cancer cell models respectively, and their source of primary cells, mouse embryonic fibroblasts (MEFs). From this model system, we created a system map for transformation to identify molecular components common between stem and cancer cells but different from primary cells. We determined regulated/activated cassettes that are unique for cancer cells by hardwiring differential expression patterns from whole genome expression array (Illumina), genomic rearrangements (copy number and structural variations) from genomic paired end tag (gPET) sequencing (Illumina), and splice forms and single nucleotide variations from RNA sequencing (RNA-seq, SOLID).

Results and Discussion: From differential expression analysis, we obtained 4 important expression patterns: stem cell specific expression, cancer cell specific expression, stem–cancer common genes with distinct expression levels or identical expression levels. Starting from the stem-cancer identical component which is responsible for the self-renewal ability of stem and cancer cells, we built maps by both stem and cancer specific networks by adding expression cassettes and mutations specific to each cell type.

Conclusion: We believe that identification and manipulation of cancer specific networks which activates self renewal will be helpful to push cancer cells towards their normal (differentiated) behavior or to kill them.

Genes Deregulated in HeLa Cells Expressing BRCA1 Missense Variants Are Involved in DNA Double Strand Breaks Repair by Homologous Recombination


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Introduction: M1775R and A1789T are two missense variants located within the BRCT domains of BRCA1 gene. The M1775R variant has been described as deleterious previously while the A1789T variant has been classified as probably deleterious for the first time by our group. To investigate the molecular mechanism that may underlie a pathogenic role for these two mutations, we compared the expression profile, obtained by microarray, of HeLa G1 cells transfected with the two variants. Compared to BRCA1 wt, the M1775R and the A1789T variants showed 201 and 313 differentially expressed genes respectively. The expression of nine genes (CDKN1A, EDDN1, GPR56, NF1B1, PML, SOD2, MRE11A, EEF1E1, OBFC2B), selected because of their involvement in neoplastic transformation, were validated by RT-qPCR. Moreover the expression level of five proteins was confirmed by Western Blotting (WB).

Materials and Methods: HeLa cells, containing an Integrated Recombination Substrate (hrpIDRGP), were transfected with siRNA for the genes MRE11A, EEF1E1, OBFC2B. The level of protein inactivation was determined by Western Blotting (WB).

We chose three out of nine validated genes (MRE11A, EEF1E1, OBFC2B) because of their involvement in DNA double strand breaks repair to be tested in an ‘homologous Recombination’ (HR) in vitro assay.

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We chose three out of nine validated genes (MRE11A, EEF1E1, OBFC2B) because of their involvement in DNA double strand breaks repair to be tested in an ‘homologous Recombination’ (HR) in vitro assay.

Results: In cells depleted for an irrelevant gene (HPRT) and in cells transfected with a Scrambled Negative Control Duplex, 40 × 10−3 of the cells were GFP positive: this frequency of recombination is comparable to the background frequency in parental HeLa cell line.
In cells depleted for BRCA1 the number of GFP-positive cells is less than $1 \times 10^{-3}$. The HR frequency in OBCF2B, MRE11A and EEF1E1 depleted cells were respectively of $14.5 \times 10^{-7}$ (SD 1.41), $22.0 \times 10^{-7}$ (SD 2.82) and $10^{-7}$ (SD ±1.0). In all cases the difference is statistically significant compared to the control-HPR-Depleted cells (P < 0.01; Dunnnett Multiple Test).

Conclusions: We conclude that knockingdown of OBCF2B, MRE11A and EEF1E1 reduces the HR rate in HeLa cells even if at lower levels compared to one produced by BRCA1 inactivation. Our results together with the knowledge obtained from literature suggest that those three genes might be involved in BRCA1-pathway.

[089] Genomic Alterations Between Recurrent and Non-recurrent Stage II Colorectal Tumors

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Background: Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer death. Stage II tumors have moderate risk of relapse after surgical resection, whereas patients with stage III have a higher chance of recurrence. Clinical risk factors for recurrence are insufficient to identify those patients with stage II CRC at high risk of relapse leading to potential under-treatment. Previous results by structural Gene Set Enrichment Analysis (GSEA) analysis using RNA expression data identified some chromosomal regions with enrichment in genes related to bad prognosis. This lead us to hypothesize that DNA aberrations could be selected and inherited because they confer malignant advantages to cells; and that these aberrations are already present in the primary colon carcinoma. So, the objective of this work is to find chromosomal alterations useful to identify those patients in stage II CRC at risk of recurrence.

Material and Methods: In our group, we are developing the COLONOMICS project in which for a series of stage II 100 normal-tumor paired samples and 50 controls we have data on RNA and miRNA expression, SNPs/CNVs, and methylation (see our webpage www.colonomics.org). Genome-wide Human SNP array 6.0 was used to compare normal vs paired-tumor at DNA level. Normalization and calibration of raw data was performed using CRMA2 and TumorBoost. For each locus, Total Copy Number (TCN) and B Allelic Frequency (BAF) were estimated in tumor samples using their respective normal tissues as reference. Moreover, an independent set of tumors had been recruited for further validation of discovered biomarkers.

Results: For each sample, Parent Specific Circular Binary Segmentation (PSCBS) method was used to identify and classify segments with significant different TCN and BAF values. Preliminary analysis based on GSEA identified the candidate region chr6q24 as putative prognostic biomarker, that was confirmed in copy number data (p-value=0.02). Further work is necessary to refine this analysis looking for differences between good and bad-prognosis tumors, by GISTIC software. Moreover, PLINK software will be used to look for those SNPs significantly associated with bad-prognosis tumors (trend test Cochrane-Armaitage).

Conclusions: This genomic data is promising to find biomarkers that distinguish between recurrent and non-recurrent CRC tumors in stage II. Further validation in an independent dataset is necessary to assess the strength of our findings.

[090] Quantitative Evaluation of the Methylation Pattern of DMRH19 and KdVMDR Regions in Patients With Apparent Epigenetic Alterations in 11p15

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Introduction: Hemihypertrophy, macrosomic syndromes and Beckwith-Wiedemann syndrome (BWS) (macrosomia, hyperinsulinism and visceromegaly) are abnormalities associated with epigenetic alterations in 11p15 and predisposition to developing embryonic tumours, especially Wilms tumour (WT). The telomeric domain of 11p15 contains two imprinted genes: the paternally expressed Igf2 encoding a foetal growth factor and the maternally expressed Igf2r that transcribes a noncoding RNA of unknown function. The expression of these genes is regulated by differential methylation of the H19 region (DMRH19) that is normally methylated only in the paternal allele. DMRH19 hypermethylation results in loss of normal expression of the maternal Igf2 gene. The centromeric domain contains the Kcnq1ot1 cluster; hypomethylation of this region causes aberrant activation of the maternal allele of this gene. KvDMDR loss of imprinting with normal Kcnq1ot1 methylation is common in 11p15. Therefore, this region is a candidate for the development of haematological malignancies as the most frequent alteration (3/11) associated with BWS and hemihypertrophy phenotypes. Five of the patients herein studied, with DMRH19 hypermethylation, are likely candidates to developing Wilms Tumour.

Materials and Methods: Epigenetic alterations in DMRH19 and KvDMDR were detected by pyrosequencing with specific primers designed with PyroMark Q24 MDx Platform and PyroMark Q24 reagents. Analyses of methylation indices were carried out with PyroMarkQ24 Software 2.0 (Qiagen). In all cases the difference is statistically significant compared to the control-HPRT-depleted cells (P < 0.01; Dunnnett Multiple Test).

Conclusions: These studies showed KdVMDR hypomethylation as the most frequent alteration (3/11) associated with BWS and hemihypertrophy phenotypes. In our group, we are developing the COLONOMICS project in which for a series of stage II 100 normal-tumor paired samples and 50 controls we have data on RNA and miRNA expression, SNPs/CNVs, and methylation (see our webpage www.colonomics.org). Genome-wide Human SNP array 6.0 was used to compare normal vs paired-tumor at DNA level. Normalization and calibration of raw data was performed using CRMA2 and TumorBoost. For each locus, Total Copy Number (TCN) and B Allelic Frequency (BAF) were estimated in tumor samples using their respective normal tissues as reference. Moreover, an independent set of tumors had been recruited for further validation of discovered biomarkers.

Results: For each sample, Parent Specific Circular Binary Segmentation (PSCBS) method was used to identify and classify segments with significant different TCN and BAF values. Preliminary analysis based on GSEA identified the candidate region chr6q24 as putative prognostic biomarker, that was confirmed in copy number data (p-value=0.02). Further work is necessary to refine this analysis looking for differences between good and bad-prognosis tumors, by GISTIC software. Moreover, PLINK software will be used to look for those SNPs significantly associated with bad-prognosis tumors (trend test Cochrane-Armaitage).

Conclusions: This genomic data is promising to find biomarkers that distinguish between recurrent and non-recurrent CRC tumors in stage II. Further validation in an independent dataset is necessary to assess the strength of our findings.

[091] Comprehensive DNA Methylation Profiling With the SureSelect Target Enrichment System

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Background: DNA cytosine methylation is a critical epigenetic modification involved in human diseases such as cancer and imprinting disorders. Various cellular processes including gene regulation, embryonic development, X chromosome inactivation, and chromatin remodeling are strongly associated with DNA methylation changes. Next-generation sequencing combined with sodium bisulfite treatment allows identification of methylation changes at single base resolution. However, whole genome bisulfite sequencing is prohibitively expensive. Additionally, many of the regions in whole genome bisulfite sequencing are in repetitive regions, and provide very little useful information. In many cases, the researcher is only interested in profiling a subset of biologically relevant regions.

Material and Methods: To address these needs, we have developed SureSelect Methyl-Seq, which combines Agilent's SureSelect Target Enrichment platform with bisulfite sequencing to detect methylation changes. Our Methyl-Seq design targets human genomic CpG sites within CpG islands/shores, promoters, known differentially methylated regions (DMRs) and previously determined regulatory regions. This comprehensive design covers 94Mb making it well suited to study cancer-, tissue-, and stem cell-specific DMRs.

Results: Here we describe the SureSelect Methyl-Seq workflow and demonstrate efficient target enrichment and precise methylation level detection. Further, we show high concordance with whole-genome bisulfite sequencing of known model systems. This method allows detection of about 7900 tissue-specific DMRs from colon, breast, brain, muscle, and liver. In tumor and normal tissues, potential novel genomic regions show dynamic changes in DNA methylation levels at single base-pair resolution.

Conclusions: SureSelect Methyl-Seq incorporates the advantage of SureSelect target enrichment technology into bisulfite sequencing and enables discovery of novel DMRs at single base resolution in cancer studies.

[092] Comprehensive Methylation Analysis of Colorectal Cancer for the Identification of New Biomarkers

L. Paré Brunet1, T. Berenguer1, D. Cordero1, M. Encuentra1, R. Sanz-Pamplona1, M. Crous-Bou1, X. Solé1, E. Guinó5, A. Lopez-Doriga6, V. Moreno1. 1Institut Català d’ Oncologia, UBS (Unidad de Biomarcadores y Susceptibilidad), L’Hospitalet de Llobregat, Spain

Introduction: Colorectal cancer (CRC) has the highest incidence and mortality rate of any tumor in Spain. No primary preventive measure has proven efficacy in reducing incidence but early detection through population screening reduces mortality. Therefore, the early detection of relevant biomarkers to improve the early detection is a research priority. The disruption of the DNA methylation profile is a major hallmark of human cancer. Aberration of normal epigenetic patterns occurs from early stages of tumor development and accumulates throughout cancer progression. The hypermethylation of the CpG islands located within the promoter regions of genes is usually associated with gene silencing and it is commonly regarded as a frequent mechanism of cancer gene inactivation. This study aimed to assess the potential usefulness of methylation biomarkers for diagnosis and prognosis in CRC.

Materials and Methods: In the present study we performed comprehensive DNA methylation profiling of 100 paired tumor-normal mucosa samples of
High-throughput Allele-specific Single Cell and Single Molecule DNA Methylation Studies for the Analysis of Multiple Candidate Loci

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Background: Neoplasia originates from normal cells that accumulate genetic alterations in certain regions including tumor suppressor genes. Alterations in tumor suppressor gene TP53 occur in 10% patients with chronic lymphocytic leukemia (CLL), and represent an important negative prognostic and predictive marker. p53 inactivation is more common in patients after therapy, compared to untreated patients. To enlight the mechanisms of p53 mutagenesis in CLL, p53 status was investigated repeatedly during the course of the disease.

Material and Methods: TP53 status was routinely analysed by functional analysis in yeast (FASAY) together with sequencing of colonies harbouring mutated template. Next Generation sequencer-based deep pyrosequencing (GS Junior, Roche), was performed in selected cases.

Results: p53 status of 248 CLL patients with intact p53 at first investigation was repeatedly analysed by FASAY. 150 (60%) of repeatedly investigated CLL patients received treatment by chemotherapy/chemoimmunotherapy between investigations. In 31 (12.5%) CLL patients FASAY detected a novel p53 mutation in the post-treatment investigation, suggesting that across, each fifth treated patient acquired p53 mutation. Retrospective analysis of samples taken before therapy was performed in 9 patients that subsequently developed TP53 mutation. Ultra-deep pyrosequencing revealed that all mutations were present as minor clones undetectable by standard techniques (proportion of mutated clone 0.24–13.0%).

Conclusions: Our results show that therapy in CLL patients may select TP53 mutations already existing as minor clones before the therapy. The detection of minor clones of CLL cells with TP53 mutation prior to therapy could be of a potential relevance for therapy choice and subsequent management of the disease relapse.

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Characterization of Oncogenesis Process of Sporadic and Familial Epithelial Ovarian Cancer (EOC)

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Background: Epithelial ovarian cancer is the fifth cause of cancer-related death in women in the western world and the most lethal of gynaecological neoplasms. The most important risk factor is a strong family history of ovarian/breast cancer, however only 5–10% of the ovarian cases are hereditary, with the majority being sporadic. Since still very little is known about the onco genesis process of those tumors this study was conducted in order to investigate the similarities and differences in patterns of genetic alterations in familial and sporadic EOC at high resolution level.

Materials and Methods: Genomic instability of 53 familial (21 BRCA1, 6 BRCA2, 26 non-BRCA1/BRCA2 cases) and 15 sporadic EOC was assessed on high resolution array-CGH (Agiol, 4x180K). Thirty-one carcinomas represented type II tumors (serous and endometroid grade 3). DNAcopy and CGHcall R packages were applied for data segmentation, while WECCA R package and Nexus Copy Number v5 for downstream analysis. SPSS v.17 was used for integration of clinical data which was available for 52 (76%) he cases.

Results and Discussion: Unsupervised hierarchical clustering did not reveal clear separation of familial and sporadic tumors based on their genomic instability level. However when we analyzed the tumors stratified according to their TP53 mutation status, the differences became more apparent. In particular BRCA mutation carriers showed a consistent pattern of greater magnitude of genomic loss, when compared to sporadic and familial non-BRCA1/BRCA2 tumors and when only high-grade tumors were considered. This may suggest that this phenomenon is a real feature of BRCA1/2 tumours and possibly related to homologous recombination impairment (HR), and does not reflect grade or histological differences and grade alone. We also identified commonly altered regions, that might be fundamental for oncogenesis of both familial and sporadic EOC tumors. Regions differentiating clusters rendered in unsupervised clustering were associated
with patients’ survival. Loss of 6q24–25 was found to be significantly correlated with better overall survival independent from other known factors such as FIGO stage or residual tumour.

**Conclusion:** Familial and sporadic EOC do not show striking differences in their genomic instability level, however there are particular copy number alterations in tumors with HR impairment, which might provide survival advantage. Our results suggest that deletion at 6q24–25 may serve as a prognostic factor in EOC, although its potential in clinical application needs to be further validated.

**[S15]** The PAI and TGFBR1 Genes Have a Significant Mutation

**Frequency at a Single Nucleotide Polymorphism (SNP) Marker Sites in Caucasian Patients With Advanced Colorectal Cancer as Compared to Healthy Controls**

M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2, M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2, M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2, M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2, M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2, M. Pauly1, B. Metzger1, M. Dicato1, A. Menzel2.

**Centre Hospitalier de Luxembourg, Recherche sur le Cancer et Maladies with patients’survival.** Lossof6q24−25wasfoundtosignificantlycorrelated to be further validated.

**Conclusion:** In the present study we identified novel p53-regulated genes involved in chromatin remodelling. In particular, the histone demethylase JMJD2B appears to be an interesting transcriptional target of p53, able to enhance cellular responses following DNA damage, thereby furthering our knowledge about the pivotal role of p53 as a tumor suppressor protein.

**[S19]** Deciphering Non-BRCA1/2 Familial Breast Tumor Heterogeneity by MiRNA Expression Profiling

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**Background:** Familial breast cancer comprises 5–10% of all breast cancers. Germline mutations in the currently known high-risk breast cancer genes (such as BRCA1/2) are common in familial breast cancer, but can explain, at best, 20–25% of the overall excess familial risk, while the remainders of cases are designated as BRCAx tumors. MicroRNA (miRNA) expression deregulation has been extensively implicated in cancer pathogenesis, and specifically in breast cancer. altered miRNA expression profiles have been associated to specific breast cancer subtypes or tumor biological features such as hormone receptor and HER2 status, metastasis or progression. But so far, no publications described miRNA expression in familial breast tumors.

**Material and Methods:** We established the miRNA expression profiles associated to familial breast tumors from different genetic subtypes, BRCA1, BRCA2 and BRCAx. We selected tumor areas from 80 FFPE samples (66 hereditary breast tumors, 10 sporadic and 4 normal breast samples). The miRNA expression was determined using LNA-based miRNA microarray, and data were analyzed by hierarchical clustering over 444 miRNA genes (>0.1); for class discovery we implemented Consensus clustering algorithm that determines an optimal number of clusters by reiteration analysis. Next, we identified miRNA signatures for each of the well defined groups and performed pathway enrichment analysis. Finally, clinicopathological data was associated to each tumor subgroup.

**Results:** Hierarchical clustering of BRCAx tumors revealed clear heterogeneity, with 4 apparent groups. Consensus clustering algorithm confirmed the existence of 4 different subgroups among BRCAx tumors (A, B, C and D). Importantly, these subgroups are maintained when performing unsupervised clustering including BRCA1/2 mutated, sporadic breast tumors and normal breast tissue. Interestingly, Group C clusters with BRCA1/2 mutated tumors, while Group A clusters along with normal breast tissue and is enriched for ER+/HER2+ tumors. Group B appears as a homogenous group of tumors, while Group D represents a very heterogeneous group, lacking a specific miRNA signature and dispersed over the cluster.

**Conclusions:** We found that miRNAs contributed to a better definition of BRCAx group of tumors, which can be subclassified into distinct groups related to specific miRNA-expression signature and different histopathological features.

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**Sunday 8 – Tuesday 10 July 2012**

**Poster Session**

**Signalling Pathways**

**[S20]** ErbB2-driven Invasive Breast Cancer Depends Cysteine Cathepsins B and L Expression

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Ablant ErbB2 receptor tyrosine kinase activation in breast cancer is strongly linked to an invasive disease. Here, the molecular basis of ErbB2-driven invasion is largely unknown. We show that lysosomal cysteine cathepsins B and L are elevated in ErbB2 positive primary human breast cancer and function as effectors of ErbB2-induced invasion. We have analyzed an MCF7 breast cancer cell line with inducible overexpression of NH2-terminally truncated, constitutively active ErbB2 (p95AN-ErbB2), and found that induction of ErbB2 expression induces activity of cysteine cathepsins B and L, demonstrating an malignant invasive phenotype on 3D invasion assays. Using an in vitro high-throughput siRNA library screen (Silencer Select Human Kinome siRNA library, Ambion) identified two known kinases downstream of ErbB2 signaling and four previously unknown kinases as crucial regulators of ErbB2-induced cysteine cathepsin expression and breast cancer cell invasiveness. Further study on the role and hierarchy of these kinases were done in different breast cancer cell lines using RNA interference, immunofluorescence, real time PCR, immuno blotting, and 2- and 3-dimensional invasion assays. With help from
bioinformatic analysis we identified 10 different putative transcription factors (TF's) and TF binding sites in the promoter regions of CTSE and CTSL1 genes. Further analysis has elucidated at least one transcription factor that binds to an ErbB2-responsive enhancer element in the first intron of cathepsin B gene (CTSB) activating the transcription of CTSB. This work provides a model system for ErbB2-induced breast cancer pro-invasive, reveals a signaling network that is crucial for invasion in vitro and defines a specific role and targets for the identified serine/threonine kinases.

[021] SET Protects Mitochondria From Apoptosis Signaling and Favors Autophagy in HNSCC Cells Exposed to a Pro-oxidant
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Introduction: Previous studies showed accumulation of protein phosphatase-2A inhibitor-2 (I2PP2A/SET) in head and neck squamous cell carcinoma (HNSCC) and indicated its action in cell death/survival signaling and response to oxidative stress. The main kinase regulated by SET is the protein kinase (HNSCC) and indicated its action in cell death/survival signaling and response to oxidative stress. The main kinase regulated by SET is the protein kinase Akt involved in the PI3K signaling pathway, which is frequently altered in HNSCC. Here we addressed potential role of SET protein in mitochondria-mediated apoptosis signaling and autophagy in the HNSCC HN13 cell line either with (siSET) or without (siCT) SET knockdown, and association with Akt phosphorylation, in presence of the pro-oxidant tert-butylhydroperoxide (t-BHP, 250 μM) with or without the PI3K inhibitor (LY 294 002®).

Material and Method: Mitochondrial and cytosolic proteins were prepared using subcellular fractionation kit (Calbiochem). Caspase activity was determined with specific fluorescent substrates. SET, Akt, phosphorylated-Akt, Bax, Bcl-2, LC3B-II and PARP proteins were analyzed by immunoblotting using respective primary antibodies and detected by peroxidase-conjugated secondary antibodies. Autophagy was estimated by detection of acidic vacuolar organelles (AVOs)/vesicular pH gradient levels with acridine orange (red fluorescence) through flow cytometry.

Results: The expression of HN13 cells to t-BHP increased levels of phosphorylated-Akt [2,14] and prevented translocation of the pro-apoptotic protein Bax to mitochondria, retained the anti-apoptotic protein Bcl-2 in the organelles, as well as sustained the cell ATP levels. SISSET in HN13 cells did not increase levels of phosphorylated-I2PP2A in response to t-BHP and minimized the mitochondrial responses to the pro-oxidant. Also LY 294.002 sensitized HN13 cells to t-BHP, suggesting the involvement of the PI3K/AKT-dependent signaling in this protective mechanism. An increase in LC3B-II and AVOs levels, without caspases activation or PARP cleavage, were observed in HN13 cells exposed to t-BHP, which is consistent with the induction of autophagy without involvement of apoptotic mechanisms.

Conclusion: SET protects mitochondria from apoptosis signaling and favors autophagy in HNSCC cells exposed to a pro-oxidant as a potential recycling mechanism of damaged cellular content. Supported by: FAPESP and CNPq, Brazil.

[025] Nrf2 Inhibition by the Coffee Constituent Trigonelline Sensitizes Pancreatic Cancer Cells to Apoptosis by Death Ligands and Chemotherapeutic Drugs
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The transcription factor nuclear factor-E2 related factor-2 (Nrf2) as a target of antioxidant therapeutic interventions is playing an established role in the chemoprevention of cancer development. However through the induction of cyto-protective and pro-oxidative genes, Nrf2 confers apoptosis protection in tumor cells, and inhibiting Nrf2 would be therefore an efficient tool in anti-cancer therapy.

Aim: The aim of the study was to investigate the feasibility of the alkaloid and coffee constituent trigonelline in inhibiting Nrf2 activity in tumours and in the therapy of apoptotic-resistance in pancreatic cancer.

Methods and Results: In the present study, three pancreatic carcinoma cell lines (Panc1, Colo357, MiaPaCa2) and the pancreatic duct cell line H6C7 were analysed for the Nrf2 inhibition effect of RP1620 and its impact on Nrf2 dependent resistance to TRAIL and anti-cancer drug induced apoptosis. Chemoresistant Panc1 and Colo357 cells exhibit high constitutive Nrf2 activity, whereas chemosensitive MiaPaCa2 and H6C7 cells display little basal but strong BHQ2-inducible Nrf2 activity and drug resistance. Trigonelline efficiently blocked basal and inducible Nrf2 activity in all cell lines, exhibiting highest effects at 0.1 μM. Along with Nrf2 inhibition, trigonelline blocked the Nrf2 dependent expression of pro-oxidative genes such as s5a and alpha5 and reduced proteasome activity in all cell lines tested. These blocking effects were absent if the cells had been treated with Nrf2 siRNA, a condition at which pro-oxidative gene expression and proteasome activity already decreased.

Most notably, trigonelline strongly sensitized all cell line for anti-cancer drug and TRAIL induced apoptosis by Nrf2 inhibition but also by downregulation of mitochondrial gene expression.

Conclusion: We show for the first time that trigonelline is significantly sensitizing pancreatic cancer cells for apoptotic stimuli by inhibition of Nrf2 and the 26S proteasome. Thus, pharmacological Nrf2 inhibition might be beneficial in improved anti-cancer therapy.

[026] Human Kinase VRK2A Regulates Cell Invasion Through Nfat Activation and Cox-2 Expression
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Background: Human VRK2A is a member of a new Ser/Thr kinase family that emerged late in evolution, VRK2A is poorly characterized, but its expression is higher in tumors and proliferative cells and it has been identified in a subgroup of breast carcinomas. The nuclear factor of activated T cells (NFKB) is a key factor regulating a variety of processes in cells and there is increasing evidence that NFKB plays a crucial role in cancer cells promoting invasion through cyclooxygenase-2 (COX-2) expression. NFKB proteins translocate to the nucleus upon phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore ionomycin (io) stimulation. In addition, an inducible phosphorylation in NFKB transactivation domain (TAD) increases NFKB transactivation activity and therefore COX-2 expression. To elucidate what kinases are involved in this phosphorylation is essential to define NFKB modulation. The regulator of calcineurin (RCAN1) protein blocks NFKB activation by direct binding to calcineurin. However, RCAN1 phosphorylation relieves this inhibitory effect.

Methods: We determined phosphorylation by in vitro kinase assays and measured transcriptional activity of NFKB and COX-2 promoter using luciferase reporter plasmids. Interactions were performing in pulldown assays. Matrigel transwell assay were used to measured cell invasion of breast cancer MDA-MB-231 cell line and migration was observed in wound healing assays.

Results: VRK2A phosphorylated NFKB N-terminal domain, enhancing NFKB transactivation activity, and interaction between VRK2A catalytic domain and NFKB regulatory domain was detected. Also, VRK2A increased COX-2 expression through NFKB pathway since mutation in COX-2 promoter NFKB binding sites decreased induction of COX-2 by VRK2A. Furthermore, VRK2A down-regulation reduced cell invasion and migration upon PMA plus io stimulation. In addition, we ruled out an indirect effect through the RCAN1 phosphorylation since VRK2A did not counteract RCAN1 phosphorylation and it was not able to counteract RCAN1 inhibition on NFKB activity.

Conclusion: These findings demonstrate the first reported role of VRK2A as an active kinase in a cell invasion context through NFKB-dependent COX-2 expression.

[027] Gene Expression of EGFR and Mina53 in Different Histological Subtypes of Lung Cancer
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Introduction: Myc-induced nuclear antigen with a molecular mass of 53kDa (Mina53) is overexpressed in various tumors and regulates several genes related to cell adhesion, metabolism, and growth factors such as epidermal growth factor receptor (EGFR). EGFR is a receptor on the cell membrane with tyrosine-kinase activity and is a regulator of proliferation, apoptosis, angiogenesis, and tumor invasion. It is found to be overexpressed in some lung cancer histological subtypes and is a target for therapy.

Material and Method: Surgically resected specimen from 99 patients (men n = 66; women n = 33, age 57.11) with lung cancer were studied: carcinoid tumors (CT) − 23, small cell lung carcinomas (SCLC) − 13, large cell neuroendocrine carcinomas (LCNEC) − 6, adenocarcinomas (AC) − 29, and squamous cell carcinomas (SCC) − 28. The histological subtype, pTNM stage, and gene expression analysis of Mina53 and EGFR in tumor and normal lung tissue were evaluated.

Results: The distribution in stages was as follows: CT − stage I 16 (70%), II 4 (17%), III 5 (22%); SCLC − I 3 (23%), II 2 (33%), III 1 (17%); AC − I 16 (55%), II 4 (14%), III 9 (31%); LCNEC − I 3 (23%), II 2 (33%), III 1 (17%); SCC − I 12 (43%), II 6 (21%), II 10 (36%). Mina53 overexpression was found in 52% (LCNEC 67%, AC 59%, SCC 50%, CT 48%, SCLC 36%), and decreased expression − in 1 case (SCC 4%). In SCLC we observed correlation between overexpression of Mina53 and lymph node metastases (p = 0.01), and between Mina53 and advanced stage (p = 0.02). Overexpression of EGFR was observed in 34% (SCC 61%, AC 23%, SCLC 23%, LCNEC 17%, CT 13%). Decreased expression of marker was observed in 22% (LCNEC 66%, SCLC 54%, CT 22%, AC 14%, SCC 7%) (p < 0.001). Correlation between EGFR and Mina53 was observed in CT and SCLC (p < 0.01).
A Functional siRNA Screening Approach to Investigate the Mechanism Underlying GPCR-mediated EGFR Transactivation in Malignancy
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Background: In a number of cell types, activation of G protein-coupled receptors (GPCRs) can lead to the ‘hijacking’ of growth factor receptor machinery (such as the epidermal growth factor receptor, EGFR) subsequently leading to the activation of cellular signalling cascades important in outcomes including cellular growth, survival, migration and invasion. GPCR-mediated ‘EGFR transactivation’ can occur with various GPCR/ligand interactions, and is involved in a number of disease states including cancer[1,2]. Our specific interest is to investigate how the angiotensin type I receptor (AT,R) is able to transactivate the EGFR and its contribution to cell growth. While a number of candidates have been implicated in this process, the molecular events underlying this process still largely remain unresolved.

Materials and Methods: We developed a stable tractable cellular model of EGFR transactivation by introducing a HA-tagged AT,R receptor into pre-malignant human mammary epithelial cells, and have functionally characterised the cells after angiotensin II (AngII) stimulation of the AT,R. From there, we have taken the novel approach of using our transactivation model to perform a high-throughput short interfering RNA (siRNA) screen of the kinome using the Drhamma siGENOME SMARTpool library to unblinded identify genes that mediate the AT,R-EGFR transactivation response. We identified 50 candidates from our primary screen, and these genes were subsequently put through a secondary siRNA screen using the Drhamma siGENOME deconvoluted siRNA library to further validate their role in EGFR transactivation. From there, we have identified a list of high confidence genes that are currently being subjected to further validation.

Results: Pre-malignant human mammary epithelial cells containing AT,R express a functional receptor capable of binding AngII at the cell surface, and also respond to AngII by initiating intracellular Ca2+ release. Furthermore, when cells are AngII stimulated, they demonstrate robust activation of EGFR and ERK1/2, blocked by the EGFR antagonist AG1478 and the AT,R blocker canakinumab. We have used our model to perform primary and secondary kinome siRNA screens to ascertain mediators of AT,R-EGFR transactivation. From our analysis, we have identified a number of candidates (such as BMX and TRIO) that have validated as secondary kinomes for AT,R-EGFR transactivation. We have also performed pre-malignant human mammary cells overexpressing EGFR and the tumour suppressor protein, Pten to determine how EGFR transactivation impacts cellular growth. The targeted genes identified from our siRNA screen may also serve as potential therapeutic targets for the treatment of diseases for which this process is important, such as cancer.

Reference(s)

Identification of Potential mTOR-kinase Splicing Isosforms
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The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, proliferation, motility and survival affected by different environmental stimuli like nutrients, hormones and growth factors. There are two distinct multiprotein complexes: TORC1 with the Raptor and TORC2 with the Rictor. Interruption of mTOR-coordinated signalling leads to pathologies including cancer, inflammation and various physiological disorders. In transformed cells, signalling through mTOR is stimulated by defects inone or several components of mTOR pathway: PI3-K, Akt, PTEN, TSC1/TSC2. An application of RT-PCR analysis of various human cell lines allow our research group to ascertain the potential mTOR isoforms. Using them – TOR-δ isoform, which have been characterized as a potential oncogene. Our current studies were focussed on asproving the existence of potential mTOR isoforms – TORγ and TORβ in malignant cells. The bioinformatic analysis of TORβ and TORδ primary structure allowed to determine the absence of several functional domains in their structure compared to a TORα molecule. Both isoforms were cloned in eukaryotic vector pcDNA3.1 and stable cell lines were obtained. In parallel, a fragment of C-terminal part of mTORδ was cloned, overexpressed and purified from bacteria cells. This recombinant protein was used for rabbit immunization and TOR-specific polyclonal antibodies generation. The application of generated antibodies allow to detect the presence in some mammalian cell lines and tissues several bands of proteins: main form TORα (29kDa), and bands similar to TORβ, TORγ and TORδ isoforms with lower molecular weight. The investigation of phosphorylation status of up-stream and down-stream effectors of mTOR (Akt, S6K1, S6 protein) in stable cell lines overexpressing TORδ and TORγ had showed the difference compared to wild type cells. Also, we detected that phosphorylation status of TORδ, like TORγ, is rapamycin sensitive, in contrast to TORβ isoform. Finally, analysis of oncogenic properties of TORβ and TORγ by soft agar colonies formation assay had demonstrated that overexpression of TORγ, but not TORβ, lead to 1.5 fold increase in colonies number.

Thereby, our recent data had demonstrated the existence of additional mTOR isoforms (TORγ, TORδ), which (have at least TORα) potential oncogenic properties. However, the existence of new endogenous forms of mTOR has to be confirmed.

Hhat a Potential New Target in Treatment of Pancreatic Cancer
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Background: Pancreatic cancer is the fifth most common cause of cancer-related death in the UK, accounting for 8100 deaths per year. It has a high incidence to mortality ratio with a five year survival rate of just ~3%, a figure that has not improved significantly in the last 25 years, despite ongoing research. The Hedgehog (Hh) signalling pathway is hyperactive in 70% of all pancreatic cancers, and provides pro-proliferative signals to cancer cells, promoting their survival. The hyperactivation of the Hh pathway provides a promising therapeutic target that could be exploited using selective compounds. Significantly, small molecule inhibitors targeting the Hh pathway are currently in clinical trials showing promising results, highlighting the importance of Hh signalling in the progression of the disease, particularly through paracrine signalling from surrounding stromal cells. However, currently all tested drugs targeted Hh signalling in the receiving tumour cell, allowing for mutations to circumvent the inhibition. We aim to use a novel complementary strategy, by targeting the production of active Hh ligands, specifically by preventing the correct post-translational modification of Hh ligands. Hh proteins require two post-translational modifications in order to be fully active and adhere to the cell surface. Specifically, they are cholesterylated on their C-terminus and palmitoylated on their N-terminus. Hedgehog acyltransferase (Hhat) is a multi-transmembrane domain protein whose only known function is to palmitoylate Hh proteins.

Materials and Methods: siRNA knock-down of Hhat in a pancreatic ductal adenocarcinoma (PDAC) cell line (PANC-1) followed by cell growth and invasion assays. Bioorthogonal (click) chemistry on PDAC cells as a palmitoylation read-out. Co-culture assays with pancreatic stromal cells expressing Hh reporter vectors.

Results: We show that knocking down Hhat by siRNA in PANC-1 cells results in the inhibition of Shh signalling and inhibits PANC-1 cell growth and invasion. Furthermore, we have developed a single well coculture assay using an immobilised pancreatic stromal cell line, to measure Hh paracrine signalling between PDAC cells and surrounding stromal cells which will be used to screen Hhat inhibitors.

Conclusion: The reduction in cell growth and invasion of a pancreatic cancer cell line provides proof of concept that Hhat is an excellent potential target against which to develop a drug to treat PDAC. The Hh in vitro reporter system developed better represents the in vivo interactions between the cancer and stromal cells, while also providing an adaptable and high-throughput platform on which to screen for specific Hhat inhibitors.

ULK1 Controls Phosphorylation of the Tumour Suppressor Protein Beclin1
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Introduction: The autophagy pathway is controlled through the mTORC1 complex by suppressing the potential mTORC1 effectors. Among them is ULK1, inhibition of the mTORC1 pathway, through nutrient deprivation or other stresses leads to activation of ULK1 and the initiation of the autophagy pathway. Autophagy also requires the formation of active Beclin1-Hvps34 complexes to promote autophagosomal

Conclusion: Mina53 can eventually be a prognostic biomarker – in SCLCs overexpression of Mina53 is associated with lymph node metastasis and advanced stage. EGFR and Mina53 are potential targets for combined targeted therapy – there is a correlation between their overexpression in CTs and SCLCs. EGFR is a target for therapy with monoclonal antibodies, so the tumors that overexpress EGFR can be considered for treatment with these drugs.
formation and maturation. Critically, Beclin1 is a known tumour suppressor protein, frequently lost in cancer.

**Material and Method:** The regulation of Beclin1 was explored using cDNA overexpression of ULK1 enzyme in HEK293 cells. Phosphorylation of Beclin1 was characterised by MS/MS and Western immunoblotting. Analysis of Beclin1 complexes was done by immunoprecipitation and FPLC analysis of native complexes.

**Results and Discussion:** We have explored the relationship between ULK1 and the hVps34 complex, in an attempt to identify the molecular mechanism that governs hVps34 regulation, during autophagy. Overexpression of wild-type, but not kinase inactive ULK1 caused a decrease in the electrophoretic mobility of Beclin1. This increase in apparent mass was due to phosphorylation, although ULK1 was not directly responsible for phosphorylating Beclin1, at least in vitro. Phosphorylation-site mapping studies revealed that Beclin1 was phosphorylated on a novel serine at it’s N-terminus, with a 50-fold increase in phosphate, but phosphorylation at other residues was not detected, despite >73% sequence coverage. In order to identify the potential upstream kinase we examined the phosphorylation of Beclin1 by DAPk and JNK, two previously reported Beclin1-regulating kinases, but neither were responsible ULK1-dependent Beclin1 phosphorylation. Finally, analysis of Beclin1-containing immune complexes from ULK1 overexpressing cells revealed that ULK1-dependent phosphorylation caused disruption of UVRAG and Atg14L association in either Beclin1 or hVps34 immunoprecipitations.

**Conclusion:** Thus, although the identity of the kinase responsible for direct Beclin1 phosphorylation at this novel N-terminal serine is not known, we have identified a novel connection between ULK1 and hVps34 regulation and report a new mechanism for regulation of the hVps34 complex during autophagy and has significant impact for the molecular understanding of Beclin1 regulation in cancer.

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**Role of GLI2 in Human Osteosarcoma and GLI2 as a New Therapeutic Target for Human Osteosarcoma**


**Background:** The Hedgehog (Hh) signaling pathway is a developmental signaling pathway, mostly inactivated in adult tissues. Aberrant activation has been found in various human tumour types, such as lung, breast, ovarian and pancreatic cancer. The Hh signaling pathway is activated by the ligand Hedgehog that causes its receptor patched to release its repression over the coreceptor Smoothened. This triggers a cascade of events in the cytoplasm leading to activation of transcription factor GLI1. The interactions between the transcription factor GLI1 and its regulators Suppressor of Fused (SuFu) and GSK3b or the role of GSK3b in activated cells are not fully understood yet. Recently the primary cilium was shown to play an important role in signal transduction. The human embryonic kidney cells, HEK293 show an interesting pattern of GLI1, SuFu and GSK3b accumulation in the centrosome, which gives rise to the basal body of the primary cilium. Since the primary cilia of these cells were undetectable we wanted to investigate if it is possible for these proteins to interact in the centrosome in the absence of a primary cilium.

**Materials and Methods:** HEK293 cells were cultured in MEM containing 10% FBS and treated with Shh protein (3 ng/ml) for 48h and with lithium chloride (LICl) (20 mM) for 24h. For immunofluorescence staining cells were parafomraldehyde fixed and permeabilized with methanol. Primary antibodies against GLI1, SuFu, GSK3b, g-tubulin, GI2 and GI3 (Santa Cruz Biotechnology, USA) and the corresponding secondary FITC or Texas Red conjugated secondary antibodies were used. The human embryonic kidney cells, HEK293 show an interesting pattern of GLI1, SuFu and GSK3b accumulation in the centrosome, which gives rise to the basal body of the primary cilium. Since the primary cilia of these cells were undetectable we wanted to investigate if it is possible for these proteins to interact in the centrosome in the absence of a primary cilium.

**Results and Discussion:** Exogenous Shh protein treatment causes a shift in protein localization, GLI1 translocates to the nucleus and SuFu remains in the cytoplasm. The amount of cells with visible accumulations of these proteins in the centrosome decreases from 80% to 15%. This suggests that the pathway is only active and functioning properly. Preliminary results reveal that GI1 and SuFu form a complex in these cells, suggesting that their interaction is independent of the primary cilium. GI2 is undetectable in these cells, while GI3 localizes to vesicles in the cytoplasm. Therefore it is likely that GI1 is the main mediator of signal transduction.

To examine the effect of GSK3b inhibition on protein localization and interactions, we treated the cells with a GSK3b inhibitor (LiCl). Treatment elevates the pathway activity, increases expression of GLI1 and PTC1 and also causes a shift in protein localization consistent with pathway activation.

**Conclusion:** Our results propose that HEK293 cells have an active Hh signaling pathway, with the regulatory processes between GI1, SuFu and GSK3b taking place in the centrosome.

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**NormaCurve, a New Tool for the Normalization of Reverse Phase Protein Array Data**

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**Background:** Reverse phase protein array (RPPA) is a powerful dot-blot technology that allows studying protein expression levels as well as post-translational modifications in a large number of samples simultaneously. Yet, correct interpretation of RPPA data has remained a major challenge for its broad-scale application and its translation into clinical research. Satisfying quantification tools are available to assess a relative protein expression level from a serial dilution curve. However, appropriate tools allowing the normalization of the data for external sources of variation are currently missing.

Here, we sought to adapt the quantification method SuperCurve in order to include normalization for (i) background fluorescence, (ii) variation in the total amount of spotted protein and (iii) spatial bias on the arrays. Using a spike-in approach, we compare different normalization methods and assess the ability of NormaCurve to properly account for these sources of variation.

**Methodology:** We investigated the ability of NormaCurve to properly account for sources of variation by comparing the performance of various normalization methods on a set of RPPA datasets. We used a set of 200 RPPA datasets from different sources, including cancer tissue arrays, cell lines and mouse models.

**Conclusions:** Our findings suggest that NormaCurve is a promising tool for the normalization of RPPA data, which should facilitate the reproducibility of RPPA data and improve the interpretation of the results obtained from these studies.
Elevated Insulin Can Reduce Effectiveness of PI3K Inhibitors – Rationale for Co-targeting the Insulin Receptor Family and PI3K

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Background: The phosphatidylinositol 3-kinase (PI3K) signaling pathway is often disrupted in cancer, and there are major drug development programs for PI3K inhibitors. In vivo, as expected, effective PI3K blockade results in hyperinsulinemia and occasionally hyperglycemia, as PI3K signaling is an important aspect of insulin signaling in many tissues. The possibility that hyperinsulinemia may lead to resistance to PI3K targeting is plausible, as in some patients PI3K blockade leads to hyperinsulinemia with normoglycemia, suggesting that elevated insulin was sufficient to restore signaling downstream of the insulin receptor to an extent sufficient to normalize blood glucose. We carried out in vitro work as a first step to determine if high insulin attenuates the consequences of PI3K inhibition and if this could be restored by the use of a small molecule inhibitor of the insulin/IGF-1 receptor kinase.

Methods: To determine the response to the drugs, cell lines were treated for 72 hrs with PI3K inhibitors GDC-0941 (a potent and selective inhibitor of class 1 PI3K) alone or in combination with an insulin/IGF-1 receptor inhibitor (BMS-754807) in presence of insulin, with proliferation assessed by MTT as an end point. Western blot analysis was also performed to examine the relevant signaling pathways.

Results: Among the cell lines tested with GDC-0941, MCF-7 cell line had the lowest IC50 (~300nM). However, when cells were treated with GDC-0941 in presence of insulin, the effect of PI3K inhibition was attenuated, and cells were able to proliferate as well as the untreated control. To overcome this, we treated the cells with a combination of GDC-0941 and BMS-754807 in presence of insulin. We observed an additive effect of both drugs eliminating the stimulatory effect of insulin. The western analysis showed that phosphorylation of AktSer473 was stimulated by insulin and inhibited by both GDC-0941 and BMS-754807. Importantly, in the presence of insulin, Akt activation was less when both inhibitors were used than with GDC-0941 alone.

Conclusion: Combining a PI3K inhibitor with insulin/IGF-1 receptor kinase inhibitor may improve antiproliferative efficacy by minimizing the process by which PI3K can restore hyperinsulinemic blockade. Extensions to in vivo experiments and the impact of varying concentrations of insulin and the inhibitors will also be discussed.

Rationale for Co-targeting the Insulin Receptor Family and PI3K

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Background: The Notch pathway functions as an organizer in embryonic development. Recent studies have shown constitutive activation of the Notch pathway in various types of malignancies. We previously reported that inhibition of Notch pathway prevents osteosarcoma growth in vitro and in vivo (Tanaka M et al. British Journal of Cancer 2010). In this report, we examined the functional role of the Notch target gene, HEY1.

Methods: Real-time PCR: To evaluate the expression of Notch pathway molecules, we performed real-time human osteosarcoma cell lines.

Results: Growth inhibition: We evaluate the effect of HEY1 and HEY2 knockdown by MTT assay. Membrane assay: We evaluated osteosarcoma cell invasion by membrane assay following HEY1 and HEY2 knockdown. Xenograft model of osteosarcoma lung metastasis: 143B cells were transfected with GFP lentiviral particles. Stably-GFP-expressing 143B cells were inoculated into the left knee joint of nude mice. Five weeks after inoculation, metastatic nodules in the lungs were evaluated by direct microscopic visualization.

Results: Over-expression of Notch pathway molecules in human osteosarcoma cell lines: We have previously reported that Notch Pathway molecules are up-regulated in osteosarcoma biopsy specimens. Real-time PCR revealed that osteosarcoma cell lines increased the expression of Notch2, Jagged1, Dll1, MAML1, HES1, HES5, HEY1, and HEY2. Knockdown of HEY1 or HEY2 did not significantly reduce osteosarcoma growth in vitro: We examined the functional role of HEY1 and HEY2 by causing the expression of HEY1 and HEY2 were up-regulated in the Notch target genes. MTT assay revealed that knockdown of HEY1 or HEY2 did not prevent osteosarcoma growth. Knockdown of HEY1 prevents osteosarcoma invasion. Knockdown of HEY2 did not prevent osteosarcoma invasion. Knockdown of HEY1 prevents osteosarcoma metastasis in vivo: Seven of 7 control 143B cells inoculated mice showed lung metastasis. On the other hand, only 1 of 7 HEY1 shRNA transfected cell inoculated mice showed lung metastasis. Knockdown of HEY1 significantly decreased the lung metastasis in vivo.
Results: We found that three circumstances are pre-requisites for the single-pathway Synthetic Lethality scenario: reversibility of pathway steps, presence of a compensatory pathway and toxicity of at least one pathway intermediate. Further modeling revealed the potential contribution of synthetic dosage lethal interactions in such a genetic system.

Conclusion: Single-pathway synthetic lethality can serve as a useful concept for developing new cancer treatment modalities acting through DNA damage.

Towards of Atlas of Cancer Signaling Networks – Basis for the Institut Curie Systems Biology Platform for Data Analysis and Interpretation

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Background: Cancer is a complex disorder that can be seen as a systems biology disease. There are numerous cell signaling mechanisms that are dysregulated. To understand the interplay of different mechanisms in the disease, systematic representation and analysis of the processes is needed.

Methods: To achieve the goal, we are in the process of creating the Atlas of Cancer Signaling Networks (ACSN), where signaling mechanisms are represented as comprehensive maps amenable for computational and mathematical analysis (http://acsn.curie.fr).

Results and Conclusions: Currently ACSN consists of four maps: cell-cycle regulation by RB-E2F, DNA repair, Cell Cycle and checkpoints, Apoptosis and energy metabolism and Cell Survival signaling networks. We will include in ACSN additional maps for EphThia-Mesenchimal Transition (EMT), Telomerese maintenance, Centrosome maintenance, DNA replication and Inflammatory processes. We have developed a Google Map-based tool NaviCell (http://navicell.curie.fr) for exploring large signaling networks. The tool is characterized by the unique combination of three essential features: (1) map navigation based on Google Maps engine, (2) semantic zooming for viewing different levels of details of the map and (3) integrated web-based blog for collecting the community curation feedbacks. NaviCell facilitates curation of molecular interactions maps by the community helping to update and maintain maps in an interactive and user-friendly fashion. We have developed a series of tools for network analysis (BioNB, OCSANA, etc), which enable structural analysis, target identification and integration and analysis of high-throughput data using ACSN maps.

Endogenous Inhibitors of Tyrosine Kinase Receptors – a Therapeutic Role for Soluble LIRI1 in Malignant Glioma

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Deregulated growth factor signaling through receptor tyrosine kinases (RTKs) is a major driving force of cancer development. In glioblastoma (GBM) the RTK genes most commonly amplified or mutated include EGFR (almost 50%), ERBB2 (8%), PDGFRα (13%) and MET (4%). The majority of GBMs that display EGFR gene amplification also express a mutant form of the receptor (EGFRVIII) that lacks exon 3 and is constitutively active.

LRIG-1 inhibits EGFR signaling by binding to the extracellular domain of the receptor, thereby inducing internalization and ubiquitination of the protein complexes. We show that LRIG-1, corresponding to the extracellular domain of LIRI1, strongly inhibited glioma growth in vitro and in vivo in patient-derived GBM xenografts. Local delivery of sLRIG1-1 to the tumor led to an almost 50% growth reduction in primary patient derived glioma xenografts, a stunning result in glioma treatment, where a growth inhibition of 5−10% is generally considered promising. Interestingly we found that sLRIG1 inhibited proliferation of glioma cells irrespective of EGFR type and expression level, and that inhibition of EGFR was not affected by sLRIG1 treatment. This suggests that the mechanism of action of sLRIG1 is not exclusively dependent on EGFR and that sLRIG1 may act as a pan-RTK inhibitor agent. Ongoing studies will identify the interaction partners of sLRIG1 and possibly the signaling pathways involved in sLRIG1-induced growth arrest.

VEGF-C Mediates RhodGDI2-induced Gastric Cancer Cell Metastasis and Cisplatin Resistance

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Introduction: Rho GDP dissociation inhibitor 2 (RhoGD2) was initially identified as a regulator of the Rho family of GTPases. Our recent works suggest that RhoGD2 promotes tumor growth and malignant progression as well as enhances chemoresistance in gastric cancer. To understand how RhoGD2 regulates gastric cancer metastasis and chemoresistance, we performed DNA microarray analysis to compare changes in gene expression by RhoGD2 overexpression and depletion. We found that the expression of several genes was changed after RhoGD2 overexpression and depletion. In this study we focused on vascular endothelial growth factor (VEGF-C), because of the following reasons: (1) it promotes tumor metastasis via an enhancement of angiogenesis, lymphangiogenesis and cancer cell invasion, (2) it confers cancer cells resistance to chemotherapy by enhancing Bcl-2 expression, all of which are similar with the phenotype of RhoGD2-overexpressing gastric cancer cells.

Materials and Methods: Gain- and loss-of-function approaches for RhoGD2 and VEGF-C were performed to explore the effect of these genes on gastric cancer metastasis and chemoresistance. The correlation of expression levels between RhoGD2 and VEGF-C was analyzed in gastric cancer patients by using tissue microarray analysis. The importance of Rac1 activation in RhoGD2-mediated VEGF-C expression was analyzed in gastric cancer cells by using small interference RNA.

Results and Discussion: RhoGD2 upregulates VEGF-C expression and secreted VEGF-C is critical for RhoGD2 and gastric cancer cell invasion and cisplatin resistance. Depletion of VEGF-C expression suppressed RhoGD2-mediated gastric cancer metastasis and sensitizes RhoGD2-overexpressing cells to cisplatin-induced apoptosis in vitro and in vivo. Moreover, RhoGD2 positively regulates Rac1 activity in gastric cancer cells. Inhibition of Rac1 expression or its activity suppresses VEGF-C expression and is associated with decreased invasiveness and increased sensitivity to cisplatin in RhoGD2-overexpressing cells.

Conclusion: RhoGD2 promotes gastric cancer cell metastasis and chemoresistance by upregulation of VEGF-C expression. RhoGD2 might be a potential therapeutic target for simultaneously reducing metastasis risk and enhancing chemotherapy efficacy in gastric cancer.

A New Chromosome 14-based Human Artificial Chromosome (HAC) Vector System for Efficient Transgene Expression in Human Primary Cells

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Background: The use of non-integrating human artificial chromosomes (HACs) in gene therapy possibly allows for safe and reliable genetic modification of human cells without insertional mutagenesis and/or unexpected oncogene activations. Although we previously demonstrated that the HAC production is long-term therapeutic erythropoietin (EPO) production in normal human primary fibroblasts (hPFs), the expression level of EPO was too low to provide medical benefits for human therapy. Thus, the next challenge for the application of this system in therapeutic purposes is to improve the transgene expression on HACs.

Materials and Methods: We newly constructed chromosome 14-based HACs and examined the effects of the telomere and promoter regions on the expression level of the transgene in hPFs.

Results and Discussions: We showed that the use of natural telomere/subtelomere and enhancers within the 5′ untranslated region of the human ubiquitin C gene greatly increased (over 1000-fold) the EPO production in hPFs. Furthermore, we demonstrated the reprogramming of mouse embryonic fibroblasts by HAC-mediated introduction of four transcription factors, and established induced pluripotent stem cells with no trace of the HACs carrying multiple expression cassettes with large genome fragments.

Conclusions: These results indicate that this HAC system could allow us to manipulate multiple transgenes efficiently in human primary cells, providing a promising tool not only for gene therapy but also for investigating genome functions in drug discoveries.

Daily Expression of Cyp19 Aromatase Gene is Altered in Mouse Leydig Tumor Cells and Clock Dependent in Male Reproductive Tract

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Background: Aromatase is an enzyme able to irreversibly convert androgens into estrogens. Aromatase was found in many tissues including male reproductive tract in mammals, where it modulates spermatogenesis and testicular development. Molecular clock is another mechanism regulating levels of reproductive hormones and maintaining spermatogenesis in a circadian manner. Both of those mechanism are linked to tumorigenesis and also can affect fertility. Recent findings revealed also that pathways critical to cell
Material and Methods: All cells were cultured in the medium under conditions recommended by manufacturer ATCC. To induce and synchronize molecular oscillator in cultured cells standard growth medium was exchanged with serum-rich medium (50%). After 2 hr serum rich medium was replaced with serum-free cell adequate medium. Male C57Bl/6J mice aged 10–15 weeks were fed ad libitum and maintained at 12:12 LD cycle. Total RNA from cultured cells and mice tissues was extracted by TriReagent and converted to cDNA. qPCR was assessed to determine mRNAs levels.

Results: Our results show rhythmic expression of core clock gene in mice reproductive tract, as well as in Leydig cultured cells. Moreover expression manner of those genes differs in immortalized and tumor cell lines. In all of studied parts of male reproductive tract of mice the expression of Cyp19 gene is rhythmic. Although expression pattern differs among investigated tissues. Cyp19 also shows rhythmic expression in Leydig cell lines, and consequently tumor cells exhibit altered expression profile compared to normal cells.

Conclusions: Our data may provide new evidence that the core molecular oscillator and aromatase Cyp19 gene are linked to mechanism of cancer development.
sites by TargetScan algorithm. We defined as alternative microRNA binding sites that sites located between two APA cleavage sites. A list of alternative microRNA binding sites has been compiled for all genes showing shortened 3’UTR expression in Ewing’s sarcoma data. The results obtained from the analysis of Ewing’s sarcoma data overlap significantly with available annotations of APA events, confirming the interest of the proposed procedure to identify candidate 3’UTR shortening events.

Reference(s)

559 A Pathway-based Design of Rational Combination Therapies
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Introduction: Cellular functions and activities are regulated by complex networks of regulatory and signal transduction pathways. Cancer diseases arise from abnormal behavior in these networks. Thus a crucial problem in biomedical research is the identification of intervention strategies that can halt pathological behaviors while minimizing side-effects. Accordingly, combinations or multi-component interventions are necessary to cope with two of the fundamental challenges in targeted therapies: redundancy and multifunctionality of biological networks [1]. On one hand, redundancy requires for several pathways to be targeted as alternate routes may be activated in response to the inhibition of a pathway. In another hand, multi-functionality with respect to physiological activity suggests that intervening at central players often causes unintended side-effects requiring the identification of alternative and possibly more distributed points of intervention [2]. Therefore systemic and efficient methods for the identification and ranking of optimal combinations of points of intervention can be very useful particularly, when addressing large networks.

Materials and Methods: We have developed OCSANA (Optimal Combinations of Intervention Strategies for Network Analysis), a freely available software for the identification and ranking of optimal combinations of points of intervention to induce the blockage of signaling from specified source nodes to specified targets. Additionally, OCSANA identifies effects with respect to non-targeted pathways (side-effects) to further optimize the combinations of intervention points. Our method is purely based on the topological structure of the signaling pathways (singed directed graph structure). The underlying algorithm is based on a classical mathematical problem, the so-called Minimal Hitting Set problem.

Results: We applied our method to identify combination therapies from our cohort of human epidermal growth factor receptor 2 (HER2)-positive breast cancer tissue samples. First we used transcriptome microarrays to compare HER2+ data with that obtained from normal breast tissue samples. We identified key players for the family of epidermal growth factor receptor (ErBb) family pathways together with less expected molecular mechanisms potentially involved in the molecular pathology of HER2+ breast cancer. With the assembled network of identified pathways and with the use of OCSANA, we identified some optimal combinations of intervention points and validated our theoretical predictions with recently published literature. These theoretical predictions validated by recent literature, provide encouraging evidence for further applications of our method for the discovery of in silico drug combination therapies.

Reference(s)

657 PIM-2-mediated Activation of the DNA Damage Response Leads to Increased Viability Following UV Radiation
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Background: PIM-2, a member of the PIM serine/threonine kinase family, is known for its pro-survival activity. Increased expression of Pim-2 was reported in several tumors, emphasizing Pim-2’s involvement in human tumor formation. However, the role of Pim-2 in the context of UV induce DNA damage, has not been determined yet. The aim of this study was to assess the potential involvement of PIM-2 in the cell’s response to UV radiation and thus to the activation of the DNA damage response.

Materials and Methods: PON-A or TET-ON inducible systems were established for expression of Flag-tagged 41kDa or HA-tagged 34kDa isoforms of PIM-2, respectively, in U2OS osteosarcoma cells. PIM-2 over-expressing cells, and controls, were exposed to UV radiation (242 nm; 50 mJ/cm²), and various DNA damage response mediators were monitored by: real time PCR, Western Blot analysis, and immunocytochemical staining. Apoptosis was determined by using the annexinV/PI method or by determining the sub-G1 phase in FACS analysis. Silencing of Pim-2 or E2F1 was performed using shRNA or siRNA, respectively.

Results: U2OS cells that were exposed to UV-radiation reacted in a significant increase in endogenous PIM-2 levels. PIM-2 over-expressing cells (either the 41kDa or the 34kDa isoforms) exhibited decreased sensitivity and increased survival following UV radiation. Over-expression of PIM-2 lead to increased levels of E2F-1, and the protective effect of PIM-2 against UV radiation was E2F-1-dependent. PIM-2 over-expression in E2F-1 silenced cells exhibited reduced viability compared to E2F-1 positive cells, following UV radiation. Moreover, over-expression of PIM-2 (both isoforms) resulted in significant increase in phosphorylation of ATM on Ser1981 (pATM), and upon UV-radiation the intensity of the p-H2AX signal (that usually appears about 3 hours after radiation) was significantly reduced compared to control cells. Both, the increased level of pATM and reduced p-H2AX signal were much less dramatic upon silencing of E2F-1.

Conclusion: PIM-2 has a protective effect against UV-radiation, an effect that is mediated by increased E2F-1 levels. E2F-1 promote ATM phosphorylation, possibly rendering the cell more ready for the coming DNA damage, as reflected by the reduced p-H2AX signal after the radiation induced damage. These findings strongly link PIM-2 family to the cellular DNA damage response mechanisms.

582 A Role for ERK Signaling in Regulation of Promoter Activity of the mPtG1 Locus in BTC3 Insulinoma Cells
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Human PTTG1 was first identified as an oncprotein found in puitive tumors and later associated with securin, a yeast protein reportedly involved in chromosome separation. Mice lacking PTTG1 are viable and fertile but males develop diabetes mellitus due to defective β-cell proliferation, among other minor problems.

RasGrf1 is a guanine-nucleotide releasing factor that enhances Ras activity. As PtG1+1, RasGrf1+2 mice also show impaired β-cell proliferation and neogenesis. A possible functional link between these two proteins is suggested, at least in retina, by the strong repression of PtG1 protein expression observed in RasGrf1 knockout (KO) mice when compared with wild type (WT) controls.

It has been reported that the PtG1 promoter interacts with Sp1, Oct-1, E2F1 and NF-Y transcription factors but it has more potential transcription factor binding sites, such as USF-1, EK-1 or GATA-1, 2 and 3, as we have seen in interanalyses using TFESEARCH ver.1.3 to search for highly correlated sequence fragments versus the TFMATRIX transcription factor binding site profile database.

Our approach to studying the relationship between RasGrf1 and control of expression of the mPtG1 promoter is based on luciferase assays transfecting a pGL-3 vector containing 2.3kb of this promoter, using a Renilla-containing vector for normalization. In addition, cells were transfected with either an empty pBK-CMV vector, or the same vector harbouring a full-length RasGrf1 insertion in it. Three different cell lines were used in this study: COS1, 293T and BTC3. Agonists used for stimulation of the cells included EGF, Ionomycin, LPA, NGF, IGF-1 and PDGF. Finally, U0126, wortmannin, SP600125, SB202190 and InSolution™ Raf1 Kinase Inhibitor II, were used as inhibitors of signalling pathways downstream of RasGrf1.

The overexpression of RasGrf1 seems to regulate mPtG1 promoter activity in a tissue- or cell type-dependent manner. In COS1 and 293T cells, where RasGrf1 is not expressed under normal conditions, its overexpression decreases this promoter activity; whereas, in BTC3 cells, it increases it. In addition, adding the U0126 MEK inhibitor in presence or absence of RasGrf1, we observed a 50% downregulation in mPtG1 promoter activity in all cell lines.

In conclusion, our results suggest a role for the ERK signaling pathway in control of mPtG1 promoter activity in all cell lines analyzed. This control may depend on Ras activation by RasGrf1 in pancreatic beta cells, where RasGrf1 is naturally expressed, but not in other cell types, where a different GEF might be responsible for Ras activation. The observed effect of MEK activity on regulation of PtG1 promoter may have an important role in tumors where miss-regulation of PtG1 expression is observed.
Activating TGF-beta Signalling Enhances the Efficacy of MAP-kinase Pathway Inhibitors in Melanoma
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Background: The RAS/RAF/MEK/ERK-MAP kinase pathway is central to cell proliferation and survival and it is deregulated in over 90% of melanomas. In approximately 30% of all cases this is due to mutations in the small GTPase NRAS, and in over 45% caused by mutations in the kinase BRAF. Although the BRAF inhibitor vemurafenib has been shown to be very effective at relapse rapidly with tumours that display resistance to BRAF inhibition through various mechanisms. An alternative approach with a broader application range is targeting the kinase MEK, which acts downstream of both, NRAS and BRAF.

Currently trialed MEK inhibitors show activity against its target and affect proliferation within the tumours, but the responses are low and incomplete. This suggests that the cytotastic effects produced by MEK inhibitors are not sufficient. However, due to dose limiting toxicity and short half-lives, drug concentrations required for cytotoxic effects cannot be achieved. Thus, identifying mechanisms that lower the threshold for pro-apoptotic activities of MEK inhibitors is crucial if we want to improve the efficacy of these drugs. Therefore, we wished to identify proteins that counteract the pro-apoptotic effects produced by MEK inhibition thus providing primary resistance to MEK-inhibitor cytotoxicity, because such proteins represent ideal targets for MEK-inhibitor combination therapies.

Material and Methods: We performed cell based assays using MEK inhibitors and RNAi approaches and performed in vivo studies in mice and zebrafish.

Results: We identified the TGF-beta antagonist SMURF2 as regulator of resistance to MEK-inhibitor induced apoptosis. Activating TGF-beta signalling via SMURF2 in melanoma cells significantly lowers the threshold for MEK-inhibitor induced apoptosis, leading to cell death in vitro within shorter time and at remarkably lower concentrations. Moreover, using different xenograft models we demonstrate that targeting SMURF2 in melanoma cells profoundly enhances the anti-tumour efficacy of MEK inhibitors in vivo.

Conclusion: We have identified a novel approach that will allow to 1. Improve the initial response to MEK inhibitors by enhancing their cytotoxic effects in resistant cells and 2. Prevent secondary acquired resistance coming form the selection for such primary resistant cells in the presence of drug.

Epigenetic Silencing of the Negative Feedback Regulator of Mitogen-activated Protein Kinase (MAPK) Signalling, DUSP5, in Colorectal Cancer
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Background: MAPK pathways transmit extracellular signals, such as growth factors or stress signals, through three-tiered kinase modules into the nucleus to initiate proliferation, differentiation or apoptosis. The magnitude and duration of pathway signalling is tightly regulated by the induction of negative feedback regulators, which include the dual-specificity phosphatase (DUSP) family of MAPK phosphatases (MKP). Signalling through the extracellular signalregulated protein kinase (ERK) pathway (MAPK/ERK pathway) is deregulated in 50% of all colorectal cancers (CRC) due to mutations in the proto-oncogenes braf or kras. Whether negative feedback regulators of the pathway need to be overcome in order to facilitate constitutive MAPK/ERK signalling in CRC, and the mechanisms by which this may occur are unknown. We postulate that one mechanism by which negative feedback regulators may be inactivated is through epigenetic silencing.

Material and Methods: DUSP gene methylation status was determined in 39 CRC cell lines using Infinium Human-Methylation27 beadchips and was confirmed by bisulphite sequencing for specific loci. Quantitative PCR (qPCR) was performed to determine DUSP5 gene expression in cell lines and human patient samples. RNA-mediated knockdown of target genes was performed by transient transfection of siRNAs into cancer cells using Lipofectamine RNAiMAX transfection reagent and a negative control siRNA transfection. The expression levels of the DUSP5 mRNA were evaluated by qPCR.

Results: Methylation array-based screening of MKP gene family members in 39 CRC cell lines identified DUSP5 as a gene frequently methylated in colon cancer. To confirm this finding the methylation status of DUSP5 promoter CpG island was analysed by bisulphite-sequencing in 19 colon cancer cell lines. This analysis revealed high DUSP5 CpG Island methylation in app. 65% of cell lines. Further, analysis of a large independent cohort of 15 such CRC cell lines revealed CNV analysis performed by transient transfection of siRNAs into cancer cells using Lipofectamine RNAiMAX transfection reagent and a negative control siRNA transfection. The expression levels of the DUSP5 mRNA were evaluated by qPCR.

Conclusion: This analysis revealed higher a DUSP5 methylation levels in tumours compared to control samples. In parallel, DUSP5 mRNA expression was significantly downregulated in colon cancers compared to normal mucosa.

Studying TGF-β Signaling Using a High Resolution, Quantitative Mass Spectrometric Approach
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Background: Members of the transforming growth factor-β (TGF-β) superfamily are critical regulators that control diverse cellular processes such as wound healing, development, differentiation and cancer. Although the TGF-β signaling pathway has been studied extensively, our knowledge of mediators and their post translational modifications is far from complete. This is evident from the fact that in addition to classical TGF-β signaling, a number of novel Smad-independent proteins and pathways are being uncovered. Mass spectrometry (MS)-based proteomics provides an unbiased platform for the in-depth study of signaling pathways. We combined high resolution mass spectrometry with stable isotope labeling of amino acids in cell culture (SILAC) to profile temporal changes on proteins and their phosphorylation on specific sites in response to TGF-β stimulation.

Methods: The SILAC-labeled keratinocyte cell line HaCaT was treated with TGF-β for 0, 6, 12, 24 and 48h for the proteome study and 5, 10, 20, 30 min and 20 h for phosphoproteomics by employing a double-triple strategy. The lysates were digested using the Filter Aided Sample Preparation Protocol (Wisiniewski et al, Nature methods, 2009). For an in-depth analysis, the peptides were fractionated using off gel separation or cation exchange chromatography with additional TiO2 based affinity enrichment of phosphopeptides. The fractions were resolved on a reverse phase column and measured online on an LTQ Orbitrap Velos. The raw data were analyzed in the MaxQuant environment and data analysis was performed in the Perseus bioinformatics module.

Results: Our study allowed quantification of more than 5,000 proteins and over 7,000 phosphosites. Established TGF-β pathway members like, Smad2, Plasminogen activator inhibitor-1 (PAI-1), TIMP3 and TGF-β activated transcription factor were found to be up-regulated on TGF-β treatment. Functional annotation analysis in the Perseus bioinformatics analysis program revealed a prominent overrepresentation of extracellular matrix protein, adhesion and motility related proteins which are positive indicators of EMT. Hierarchical clustering of the regulated proteins showed strong enrichment of the Mini Chromosome Maintenance complex (MCM), involved in DNA damage, and other replication/DNA binding related proteins. Rigorous statistics based filtration of SILAC results in the Perseus environment allowed us to identify several novel candidates which could function as EMT regulators. Of these, we validated OCIA domain containing protein 2 (OCIA2D) using shRNA based silencing followed by phenotypic assays for markers like Fibronectin and PAI-1. We are also validating phosphoproteins which could serve as major players in alternate pathways of TGF-β signal transduction.

Conclusions: This global study provides the largest quantitative dataset for TGF-β induced EMT to date. The ongoing validation of novel targets will contribute to our understanding of this important pathway.

Mathematical Modeling of Bladder Tumorigenesis
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Introduction: In bladder cancer, two types of tumors have been identified: the FGFR3 mutated tumors and the FGFR3 non-mutated tumors. The tumoral progression seems to be different in these two types of tumors, the FGFR3 mutated tumors being associated to less invasive tumors (also referred to as Ta pathway) and the FGFR3 non-mutated samples being associated to more invasive tumors (also referred to as the CIS pathway). Using mathematical modeling and statistical analyses on tumor data, we characterize two tumor invasiveness models for the context of two major cellular processes: cell cycle entry and apoptosis entry.

Methods: We built two networks, the first one is a descriptive reaction network showing the molecular links between RB and p53 pathways and the inactivation of the mutated cell line, and the second one is an influence network derived from the first reaction map. The reaction network includes 56 species (14 proteins, 14 mRNA, 13 genes), 4 inputs (DNA damage, EGFR, FGFR3, and TGFβ) and 64 reactions. The corresponding influence...
network was reduced to 14 species, 4 inputs and 3 outputs (apoptosis, proliferation and growth arrest).

**Results:** A mathematical model based on formal logic was translated from the influence network. The dynamical model accounts for diverse phenotypes of both normal and mutant cells found in the literature in response to growth and DNA damage signals. Moreover, the model verifies the hypotheses on how a cell becomes invasive in bladder tumors, through which signaling pathways and with which type of alterations or mutations invasiveness is associated in two different conditions: the FGF瑞 mutated cells and the FGF瑞 non-mutated cells. We also performed exploratory and differential analyses on expression data for the genes of the network and identified genes that play a role in the different steps of bladder tumorigenesis. The results confirmed known facts about bladder tumors and proposed new directions in extending the network description.

**Conclusion:** The combination of mathematical and statistical analyses improves our understanding of the diverse alterations observed in the two types of tumors and their importance in invasiveness in bladder tumors.


**Background:** Phytochemicals are recognised as an important source for cancer chemopreventive agents. Andrographolide is an active ingredient of the traditional Andrographis paniculata, which exhibits significant anticanic activity. Despite its impressive biological activities, the major drawback is the poor water solubility making it difficult to prepare formulations for clinical use. Transformation of andrographolide into an ester derivative (AG-4) at C14 hydroxyl improved its solubility and anti-cancer activity. In this study, we sought to delineate the apoptotic and autophagic response contributed to the anti-leukemic effect of AG-4.

**Material and Methods:** Cytotoxicity of AG-4 was evaluated in U937 cells by MTS-PMS assay along with flow cytometric measurement of reactive oxygen species using CMH2DCFDA, thiols estimation by using CMFDA, intracellular Ca++ by using Fluo4-AM, phosphatidylserine exposure by Annexin V-FITC and mitochondrial membrane potential by using JC-1. Role of pro- and anti-apoptotic proteins were evaluated by western blot analysis. Caspase activity was measured spectrophotometrically. Occurrence of autophagy as detected by AVOs formation was confirmed by transmission electron microscopy and fluorescence microscopy and Atg protein expressions by western blot analysis. Changes in nuclear morphology were detected by confocal microscopy, evaluation of DNA degradation by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay and cell cycle analysis by flow cytometry.

**Results:** AG-4 exhibited an IC50 of 5.4±m and induced apoptosis as evidenced by enhanced Annexin-V positivity. This involved increased ROS and decrease in thiol resulting in redox imbalance; elevated levels of intracellular Ca++ coupled with loss of mitochondrial membrane potential and altered pro- and anti-apoptotic protein levels. This resulted in cytochrome c release and caspase activation. Furthermore, AG-4 stimulated autophagy, which was evidenced by formation of AVOs, processing of LC3-I to LC3-II and altered Atg and Beclin 1 expression levels. Eventually, AG-4 induced DNA strand breaks confirmed by an increase in sub G0/G1 population.

**Conclusions:** Therefore, the present study identifies a new target and a new mechanism for the anti-leukemic activity displayed by AG-4 underscoring its use as a therapeutic agent against leukaemia.


**Background:** Reactivation of the Hedgehog (Hh) signalling pathway is associated with liver injury, fibrosis and hepatocellular carcinoma (HCC) development. Hh has been studied extensively using biliary but not hepatocellular liver injury models. Therefore, the aim of this study was to develop a model of proto-oncogenic liver injury in vivo using thioacetamide (TAA) treatment in mice, which mimics human liver disease progression.

**Materials and Methods:** Male C57Bl/6 mice (n = 5–10/group) were treated with thioacetamide (100 mg/kg), rising in dose by 100 mg/kg every 4, 8, 20, and 40 weeks, and treated with thioacetamide (100 mg/kg, 40 weeks) and LAMP3 has already been associated with an increased metastatic potential in breast cancer cell lines, and through mining of available CHIP-seq data from other cell systems. Of the 15 chosen targets, we uncovered five (LAMP3, ETV7, UNC5B, PRDM14, and TLR4) that are clearly involved in breast cancer cell lines, and through mining of available CHIP-seq data from other cell systems.

**Results:** To specifically investigate cooperative interactions between NF-kB and p53 on gene expression changes, we performed a genome-wide transcriptome analysis in the breast-cancer derived MCF-7 cells following single or combinatorial treatments with the chemotherapeutic drug doxorubicin and the NF-kB inducer TNF-alpha. Bioinformatics research supported the selection of combination target genes and RT-qPCR, RNAi experiments and chemical inhibition were carried on to validate our predictions.

**Conclusion:** Our bioinformatics study of the microarray data revealed potential synergistic effect of the combinatorial treatment for nearly 350 upregulated (and about 100 repressed genes). Notably, on chIP analysis applied to these gene groups revealed several players of the epithelial mesenchymal transition and cell migration. We focused the validation experiments on 15 upregulated genes and the direct involvement of p53 and NF-kB on their expression. In silico analysis of the potential target genes’ promoter sequence identified both p53 and NF-kB responsive regions where cis-mediated interactions of these TFs may occur, a result that has been followed up by CHIP assays for p53 and p65 occupancy in MCF-7 cells, and through mining of available CHIP-seq data from other cell systems. Of the 15 chosen targets, we uncovered five (LAMP3, ET7V, UNC5B, NTN1, and PLK3) that are clearly involved in synergistically doxorubicin and TNF-alpha treatment in MCF-7 breast cancer cells. Overexpression of LAMP3 has already been associated with an increased metastatic potential for particular cancers, and ET7V acts as a regulator of cell proliferation. We propose that the functional crosstalk between the p53 and NF-kB TFs can lead to the activation of specific gene expression programs that may impact on cancer phenotypes. Given that most tumors carry an activated NF-kB pathway, and a significant proportion of breast cancers, particularly the luminal subtype, maintains wild type p53, the uncovered crosstalk could potentially modify the efficiency of cancer therapy.
**Molecular Mechanisms in Interleukin-6-induced Vascular Endothelial Growth Factor-C Expression in Lymphatic Endothelial Cells**

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**Introduction:** Tumor metastasis is mainly attributable to angiogenesis and lymphangiogenesis. The more recent concept is that lymphangiogenesis is suspected as being of greater importance in relation to metastatic spread of tumor. A member of the vascular endothelial growth factor (VEGF) family, VEGF-C has been mentioned as the best lymphangiogenic factor, binds with VEGF receptor-3 (VEGFR-3), which is specially expressed only in lymphatic endothelial cells (LECs). On the other hand, the levels of Interleukin-6 (IL-6) are highly elevated in various types of tumors and IL-6 has been shown to increase tumor lymphangiogenesis through VEGF-C induction in tumor cells. However, the underlying mechanisms of IL-6-induced VEGF-C expression in LECs still need to be further clarified.

**Material and Method:** We investigate the signaling cascade involved in IL-6-induced VEGF-C expression in LECs. Phosphorylation status of the signaling molecules including focal adhesion kinase (FAK), Src, p38, ERK and Signal transducer and activator of transcription 3 (STAT3) were examined by immunoblotting. Transcription factors such as C/EBP\(\beta\), Sp1, and NF\(\kappa\)B activated by IL-6 were determined using reporter assay. Chromatin immunoprecipitation (ChIP) assay was also used to determine whether C/EBP\(\beta\), Sp1, or NF\(\kappa\)B binds to VEGF-C promoter region in LECs exposed to IL-6.

**Results and Discussion:** The VEGF-C mRNA and protein levels were elicited in LECs exposed to IL-6. IL-6 time-dependently induced FAK, Src, STAT3, p38 and ERK phosphorylation. IL-6-induced Src, p38, STAT3 and ERK phosphorylation was attenuated in the presence of P22, a Src inhibitor. IL-6 increased VEGF-C promoter luciferase activity. C/EBP\(\beta\)-, Sp1, and X-box luciferase activities were also increased in LECs exposed to IL-6. Results from ChIP analysis further showed that C/EBP\(\beta\), Sp1 and p55 binding to the VEGF-C region were increased after IL-6 exposure. 

**Conclusion:** We report a Src-mediated p38, STAT3 and ERK activation resulting in C/EBP\(\beta\), Sp1, and p55 binding to the promoter region of VEGF-C and subsequent VEGF-C expression in LECs exposed to IL-6.

**663** Modulation of AP-1, NF-\(\kappa\)B and STAT Transcription Factors by Methoxylated Thioistilbens Derivatives in Human Squamous Carcinoma A431 Cell Line

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**Background:** Resveratrol (RES), naturally occurring stilbene derivative possesses a variety of potential anticarcinogenic activities including inhibition of the proliferation of several cancer cell lines as well as modulation of transcription factors involved in cancer signaling. Low bioavailability and solubility limit application of the compound as chemo preventive oranticancer agent. Therefore the attempts are made to find novel resveratrol’s derivatives, deprived of its drawbacks, but possessing similar biological activity. Our previous study showed that insertion of thiomethyl group increased the cell growth inhibition potential of MCF7 and MDA-MB-231 breast cancer cell lines. In this study we assessed the modulation of AP-1, NF-\(\kappa\)B and STAT transcription factors in human cutaneous squamous carcinoma cell line A431. These transcription factors are involved in abnormal signaling observed in many tumors and therefore are attractive targets for potential anticancer or chemopreventive compounds.

**Material and Methods:** A431 cells has been cultured at 70-80% of confluency in Dulbecco Modified Eagle's Medium with 10% FBS. To assess thiostilbens’ cytotoxicity (8 derivatives) the standard MTT test was used (concentrations ranging from 0.5 to 100\(\mu\)M). Activation of AP-1, NF-\(\kappa\)B, and STAT was evaluated in the nucleus of the cells incubated with the tested compounds for 72 hours using Transcription Factor ELISA kits (TransAM, Active Motif, USA), with consensus site oligonucleotides specifically binding activated transcription factor subunits.

**Results:** Compared to RES its thiomethyl analogs exhibited similar growth inhibition of A431 cells, however compounds with three methoxy residues, 3,4,5-trimethoxy-4'-thiomethyl-trans-stilbene (S7) and 2,4,5-trimethoxy-4'-thiomethyl-trans-stilbene (S8) were significantly more cytotoxic than the parent compound. Only RES reduced the activation of NF-\(\kappa\)B decreasing binding of its active subunits p55/p50 to consensus site oligonucleotides, whereas its thiomethyl derivatives did not show significant effect on this transcription factor. Similarly, only RES inhibited AP-1 activation. In contrast compounds S7 and S8 stimulated AP-1 subunits, c-Jun and c-Fos binding activity to their consensus sequence. However, thiostilbenes derivatives designated S1, S2 and S7 reduced the activation of STAT, which was not affected by RES.

**Conclusions:** The results show that chemical modification of resveratrol with thiomethyl residue does not always increase the transcription factors inhibitory activity. In case of S7, the compound with three methoxylated residues, the cytotoxic effect may be attributed to the capability to inhibit STAT activation. Since constitutive activation of STAT occurs in squamous cell carcinoma this compound may be considered as the promising anti-cancer agent.

**664** Molecular Mechanisms in Simvastatin-induced HCT116 Colorectal Cancer Cell Apoptosis

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**Introduction:** Elevated level of survivin, a member of inhibitors of apoptosis protein (IAP), is often found over-expressed in human cancers, including colorectal cancer, and has been implicated in the development and progression of tumorigenesis. Because of survivin plays a central role in cell division and also act as a suppressor of apoptosis, it thus represents a potential molecular target in colorectal cancer management. Stalins, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors with cholesterol-lowering properties, were recently shown to exhibit anticancer effects in a variety of tumors. However, the molecular mechanisms underlying staliens-induced cell death in cancer cells remains to be elucidated.

**Material and Method:** In this study, we explored the underlying signaling mechanisms in simvastatin-induced survivin downregulation and cell apoptosis in HCT116 colorectal cancer cells.

**Results and Discussion:** Simvastatin decreased cell viability and induced cell apoptosis in HCT116 cells. These results are associated with the modulation of p21 and survivin. Survivin knockdown using a survivin small interfering RNA strategy also decreased cell viability and induced cell apoptosis in HCT116 cells. Survivin promoter luciferase activity was decreased while p21 promoter luciferase activity was increased in cells exposed to simvastatin. Simvastatin-decreased cell viability was restored in the presence of pifithrin, a p21 inhibitor. Simvastatin induced p53 phosphorylation and acetylation in a time-dependent manner. In addition, simvastatin activated p38 mitogen-activated protein kinase (p38MAPK) and inhibitor of p38MAPK signaling abrogated simvastatin’s effects of increasing p53 and p21 promoter luciferase activity. Survivin promoter luciferase activity in the presence of simvastatin was restored by p38MAPK inhibitor. Furthermore, Sp1 binding to the survivin promoter region decreased while p53 binding to the promoter region increased after simvastatin exposure.

**Conclusion:** We report a p38MAPK-mediated downregulation of survivin and its functional correlation with p53 increased HCT116 colorectal cancer cell apoptosis in the presence of simvastatin.
**Anti-angiogenic Mechanisms of PPemd 26, a Novel Anthraquinone Derivative**

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**Introduction:** Angiogenesis contributes to ischemic, inflammatory, infectious and immune disorders and also occurs during numerous malignancies. It is a balanced process that involves proliferation, migration, differentiation and tube formation of endothelial cells. As a critical factor in inducing angiogenesis, vascular endothelial growth factor (VEGF) has become an attractive target for anti-angiogenesis treatment.

**Materials and Method:** In an effort to develop novel inhibitors to block VEGF signaling and angiogenesis, we selected PPemd26, an anthraquinone derivative, and investigated its inhibitory mechanisms in human umbilical endothelial cells (HUVECs).

**Results and Discussion:** PPemd26 concentration-dependently inhibited VEGF-induced proliferation, migration and tube formation of HUVECs. PPemd26 also suppressed VEGF-induced microvessel sprouting from aortic rings ex vivo and suppressed new vasculature formation in implanted matrigel plugs in vivo. In addition, PPemd26 inhibited VEGF-induced phosphorylation of Akt, focal adhesion kinase (FAK), ERK and Src. Using xenograft tumor angiogenesis model, PPemd26 markedly attenuated tumor-associated angiogenesis. Taken together, our findings suggest that PPemd26 inhibits VEGF- and tumor cells MDA-MB-231-induced angiogenesis. Downregulation of VEGFR2-mediated signaling may contribute to its anti-angiogenic actions.

**Conclusion:** Results of this study suggest the anti-angiogenic activity of PPemd26 and it may be a potential drug candidate in anti-cancer therapy.

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**The Biological Connection Markup Language – a Data Format to Visualize, Annotate and Analyze Biological Pathways**

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**Introduction:** One of the most challenging goals of modern biology is to decipher the complexity of cell systems. Efforts have been conducted in the field of systems biology, as genes, proteins and metabolites are connected in complex and finely regulated networks. A number of initiatives emerged to model this complexity, such as KEGG and Reactome. Current efforts are either towards graphical representation, with the Systems Biology Graphical Notation (SBGN) the most comprehensive example (Le Novère et al., 2009) or machine-readable formats which are meant to be interpreted by computer programs.

The Biological Connection Markup Language (BCML) we developed, is a data format to model pathways according to the SBGN specification, allowing a fully dynamic representation useful for both the biologist and bioinformatician.

**Materials and Methods:** An XML schema representing the SBGN Process Description was implemented following the specification. Optional extensions were written for additional features.

To test the the format, a BCML representation of the toll-like receptor 3 (TLR3) pathway was retrieved from DC-ATLAS (Cavaleri et al., 2010). Then, a data set of dendritic cells (DCs) stimulated with poly (I:C) was downloaded from Array Express and analyzed using the BCML file: the Fisher’s Exact Test and impact analysis (Tarca et al., 2009) were used.

**Results and Discussion:** BCML supports the complete SBGN standard and optional extensions to store information of biological relevance, such as the tissue and cell type where the evidence was proven. Pathways can be customized by excluding or including elements using these information. In order to test this format, we used the BCML definition of the TLR3 pathway in a public data set. Results showed p-values of one order of magnitude lower if non-cell-specific elements were removed. These findings indicate that better analysis of pathways needs to use already existing knowledge. Application of this method in cancer would allow to separate common processes from the highly specific ones, an effort useful both to the biologist, to visualize the excluded interactions and to the bioinformatician to adjust the analysis methods.

**Conclusion:** BCML provides a convenient and precise way to represent biological pathways. Its dynamic nature makes it an important tool for the dissection of complex biological problems.

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**TCTP Expression in Lung Cancer Cells Was Dependent on mTOR**

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**Background:** Translationally controlled tumor protein (TCTP), also called histamine releasing factor, is highly conserved in various species such as yeasts, parasites, fruit flies, and mammals. It is almost ubiquitously in mammalian tissues. TCTP is known to be involved in cell growth, cell proliferation, apoptosis, and malignant transformation. Studies have reported that the level of TCTP is higher in human tumors than in normal tissue, and TCTP is down-regulated during tumor reversion. In our previous study, it was found that the level of TCTP was markedly high in lung tissues of Korean lung cancer patients and in lung cancer cell lines compared to normal controls. However, the signaling pathways that contribute to high expression of TCTP in lung cancer are still unknown.

**Materials and Method:** Lung cancer cells were treated with rapamycin, PP242, MG132, CBL, ALLN, and cyclopiazin 9, and Western blot, RT-PCR, and Northern blot were performed. Down regulation of some genes was accomplished by siRNA transfection. The phosphorylation sites important for regulating TCTP expression were confirmed by ectopic expression of histagged phosphorylation mutants of TCTP.

**Results and Discussion:** PP242 represses human K562 Cells Acting as a Transcriptional Modulator


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**Introduction:** p21 (p21 herein after) is a member of the Cip/Kip family of inhibitors of cell cycle progression. The first discovered p21 function and so far its best studied biochemical activity was the inhibition of cyclin-dependent kinases. However, other studies have shown that p21 has other functions such as inhibition apoptosis induced by DNA-damaging agents and induction of senescence or differentiation. Besides, p21 has been implicated in the control of transcription by direct association and modulation of transcription factors. Nonetheless, there is little information on the biological significance of p21-dependent regulation of gene expression and to what extent it is linked to effects on the cell cycle. To delineate the roles of p21 in transcriptional control we studied the gene expression changes using human leukemia cell line (K562) with inducible p21 expression (Kp21).

**Materials and Methods:** K562 gene expression profile was performed by using an Affymetrix HG-U133A chip. Protein and mRNA expression were measured by Western-blot and RT-qPCR respectively. Chromatin immunoprecipitation (ChIP) assays were performed to study the p21 and CDK2 binding to the transcription start site of cell cycle genes.

**Results:** We found that p21 rapidly and strongly repressed the mRNA levels of a number of genes involved in cell cycle and mitosis. One of the most rapidly down-regulated genes was CCNE2 (cyclin E2 gene). Mutational analysis in K562 cells showed that the N-terminal region of p21 is required for repression of gene expression of CCNE2 and other genes. Moreover, Chip assays indicated that p21 was bound to human CCNE2 gene in the vicinity of the transcription start site. p21 was also found on the promoter of other three down-regulated genes but not in control genes not regulated by p21. Bioinformatic analysis revealed that the CDE motif is present in most of the promoters of the p21-regulated genes.

**Conclusions:** p21 exerts a repressive effect on a relevant number of genes controlling cell cycle. Thus, p21 activity as inhibitor of cell cycle progression would be mediated not only by the inhibition of CDKs but also by the transcriptional down-regulation of key genes.

**Expression of E-cadherin and Vimentin in Epithelium-Mesenchymal Transition (EMT) in Penile Carcinoma**

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**Background:** Penile cancer is rare disease in developed countries. In Brazil, it is considered a serious health problem. Lymph nodes metastasis is the main prognostic factor; however, clinical and laboratory evaluations of it are
still unreliable. Therefore, molecular markers have been pursued. Epithelial-mesenchymal transition (EMT) is a common phenomenon in carcinomas and it is a mechanistic mechanism through which breast cells may evade the adjacent stroma. The aim of this study was to analyze EMT phenomenon through e-cadherin (ECAD) and vimentin expression using immunohistochemistry, and correlate those findings with known clinicopathological features and survival of penile carcinoma patients.

Material and Methods: 91 samples of patients with no history of cancer and 151 samples of penile carcinoma were submitted to immunohistochemistry for ECAD expression in the non-neoplastic mucosa (NNM), superficial central portion of tumors (SCPT) and in the tumor invasion front (TF); vimentin expression was evaluated regarding the intensity of cytoplasmatic staining and percentage of stained neoplastic cells.

Results: Loss of ECAD expression in the TF is significantly higher than in SCPT or in adjacent NNN. Also, the loss of ECAD expression in the TIF correlated with histological grade (p < 0.0001), infiltrative pattern of tumor growth (p < 0.0003), lymph nodes metastases (p < 0.0067), perineural invasion (p = 0.0284) and vascular invasion (p = 0.0425). In SCPT, loss of ECAD expression associated with infiltrative growth (p = 0.0116) and perineural invasion (p = 0.0225). Vimentin expression is associated with histological grade (p < 0.0001), infiltrative growth (p < 0.0001), stroma invasion (p = 0.0246) and vascular invasion (0.0003). Independent prognostic risk factors for cancer specific survival estimated by Cox regression were gain of vimentin expression (RR = 7.676; 95% CI [2.10 – 18.346]) and presence of lymph nodes metastasis (RR = 2.761; 95% CI [1.305 – 5.774]). For death not due to cancer, independent prognostic factors were expression of vimentin (RR = 2.302; 95% CI [1.405 – 3.772]), presence of lymph nodes metastasis (2.370; 95% CI [1.366 – 4.113]), and perineural invasion (RR = 1.841; 95% CI [1.073 – 3.159]).

Conclusions: Loss of ECAD expression is more frequent in tumor invasion front and it is associated with classical poor prognostic indicators such as poorly differentiated tumors, infiltrative pattern of tumor growth, perineural and vascular invasion. EMT phenotype defined by loss of ECAD and gain of vimentin expression is associated with all poor prognostic factors, including worse cancer specific and overall survival.
Results: Comparison of the gene expression pattern in normal lung tissue of lung ADCA patients with that of non-lung cancer patients with a lung metastasis pointed to a transcriptional signature enriched for genes involved in the extracellular matrix (ECM)-receptor interaction and in focal adhesion. In particular, several genes encoding collagen chains were upregulated in the non-involved lung of patients with lung metastasis as compared to that in lung ADCA patients. Since increased levels of stromal collagen may result in a more invasive tumor phenotype with increased lung metastases, the higher levels of collagen gene expression found in non-involved lung tissue of the lung metastasis patients might reflect a higher propensity to metastatic invasion.

Conclusion: Transcriptional profile analysis of normal lung tissue identified an ECM-receptor interaction pathway that distinguished patients with lung ADCA from patients with a lung metastasis of a non-lung cancer. Nine of 16 genes identified in this pathway encode collagen chains, suggesting that individual variations in ECM organization may affect risk of metastatic spread to the lung.

[076] Signaling Molecules as Biomarkers in Brain Tumors
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Introduction: Signaling pathways play a major role in pathogenesis of glioma, the most common and lethal brain tumor in adults. The evolution of brain tumors results from multiple alterations in signaling pathways. Monitoring the array of signaling molecules aims to provide a better understanding of the events in brain tumor progression and may lead to possible identification of biomarkers and therapeutic targets.

Material and Method: Signal transduction molecules, cytokines and their receptors, were determined using xMAP® array technology in tumoral, peritumoral and primary tumor cells culture lysates.

Results and Discussion: Expression levels of signaling molecules were significantly increased in glioblastoma compared to peritumoral tissue. Cell culture established from patient's tissue revealed enhanced levels of expression for all markers. These results were in correlation with cytokine and growth factor panels, also with the growth factor receptors.

Decreased expression level of signaling molecules was observed using PI3K inhibitors on primary tumor cell culture established from patient's tumor. This findings suggest that the PI3K pathway could be used as target therapy in brain tumor.

Conclusion: x-MAP® array can be used for a rapid and efficient method in the discovery of key signaling molecules, cytokines and their receptors as biomarkers in brain tumor diagnosis. Among the advantages, there are screening for molecular biomarkers, identification of potential therapeutic targets and monitoring therapy.

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[077] HER3 Expression in Human Breast Carcinomas is Associated With Tumor Size and Estrogen Receptor Status
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Background: HER3 is a type I transmembrane glycoprotein that is a member of the HER family of tyrosine kinase receptors. The role of HER signalling in breast cancer has focused primarily on HER1 and HER2, but it is becoming increasingly clear that HER3 also have an important role to play in this disease. In this study we focused on HER3 prognostic value and role in breast cancer through the analysis of 147 cases of breast carcinomas and evaluated HER3 expression and its correlation with both clinicopathological features (histological grade, tumor size, lymph node status) and molecular biomarkers (ER, PR, HER2 and Ki67).

Material and Methods: Tumor samples from 147 primary breast carcinomas were collected from the archives of the Pathology Department of our institution from 2005 to 2009. HER3 immunohistochemistry (IHC) was performed in whole sections from formalin fixed paraffin embedded blocks; expression was graded by two observers and tumors with a 2+3+ score were considered positive. Correlation between HER3 membrane staining expression, with usual pathological factors and other conventional biomarkers was analyzed using Chi-Square and Kruskal–Wallis statistical tests. Survival was assessed by the Kaplan–Meier method and compared between groups using the Mantel-Cox log rank test.

Results: Comparison of the gene expression pattern in normal lung tissue of lung ADCA patients with that of non-lung cancer patients with a lung metastasis pointed to a transcriptional signature enriched for genes involved in the extracellular matrix (ECM)-receptor interaction and in focal adhesion. In particular, several genes encoding collagen chains were upregulated in the non-involved lung of patients with lung metastasis as compared to that in lung ADCA patients. Since increased levels of stromal collagen may result in a more invasive tumor phenotype with increased lung metastases, the higher levels of collagen gene expression found in non-involved lung tissue of the lung metastasis patients might reflect a higher propensity to metastatic invasion.

Conclusion: Transcriptional profile analysis of normal lung tissue identified an ECM-receptor interaction pathway that distinguished patients with lung ADCA from patients with a lung metastasis of a non-lung cancer. Nine of 16 genes identified in this pathway encode collagen chains, suggesting that individual variations in ECM organization may affect risk of metastatic spread to the lung.

[077] FOXO3a Represses VEGF Expression Through FOXM1-dependent and -independent Mechanisms
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Background: VEGFs and their receptors are essential for breast cancer carcinogenesis, angiogenesis and metastasis. VEGF signals through different pathways including the PI3K/AKT, the p38-MAPK, and Raf however, the molecular mechanisms regulating VEGF expression in cancer cells are not fully understood. A cDNA microarray study suggested that VEGF is a potential negatively regulated target of FOXO3a. In this study we tested and examined the underlying mechanism of VEGF regulation by FOXO3a and FOXM1.

Material and Methods: The human breast carcinoma cell lines BT474 and SKBR3 as well as MDA-MB-231 were treated with lapatinib. The levels of FOXO3a, FOXM1 and VEGF were assessed at protein level by western blotting and transcriptionally by real-time quantitative PCR analysis.

Luciferase reporter assays were used to study the promoter regulation of VEGF by FOXO3a and FOXM1. CHIP and oligonucleotide pull-down assays were used to show that both FOXO3a and FOXM1 bind to the VEGF promoter. The association between FOXO3a, FOXM1 and VEGF was validated by immunohistochemistry in breast cancer patient samples.

Results: Using the lapatinib sensitive breast cancer cell lines BT474 and SKBR3 as models for FOXO3a activation, we showed that FOXO3a represses while FOX1M activates VEGF expression at the gene promoter level. We demonstrated that both FOXO3a and FOXM1 compete for the same binding site on the VEGF promoter. Furthermore, FOXO3a also represses FOXM1 expression at the translational level. Consequently, FOXO3a can repress VEGF expression indirectly via regulating FOXM1 expression. Further analysis revealed that FOXO3a recruits HDACs to remodel chromatin and repress transcription.

Conclusions: These results unravel a novel mechanism by which FOXO3a is able to repress gene transcription.

[078] The Role of RNA Interference-based Regulation of Gene Expression in Cancer Cells Exposed to Ionizing Radiation
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Background: Ionizing radiation induces a plethora of changes in cells, including cell-type specific alterations in the transcriptome. One of the crucial regulatory events involves RNA interference, a mechanism which utilizes small double stranded microRNAs with the ability to control the translation efficiency and degradation rate of transcripts in order to maintain a steady state in the complex intracellular regulatory processes. Ionizing radiation can disrupt this natural balance by pushing cells into distinct states and/or inducing apoptosis and necrosis depending on the cell line, the conditions, and the radiation dose. A detailed study of the effects of ionizing radiation on RNA interference mechanisms in human cells may provide insights into the molecular background of cellular stress response mechanisms.

Materials and Methods: miRNA and mRNA levels were measured with Affymetrix HG-U133A and Agilent SurePrint G3 Human v16 mRNA microarrays in four human cell lines (107, HCT116, Me45 and HCT116, Me45 exposed or not to 4 Gy of X-radiation. miRNA-microRNA interaction sites were identified using miRanda and TargetScan bioinformatic approaches.

Results: Transcripts up-regulated 12 h after exposure to ionizing radiation show significantly (p-value <10^-13) larger numbers of motifs targeted by miRNAs in Me45 and HCT116 wt cells. Most of the target sites in the up-regulated transcripts correspond to a group of miRNAs showing a significant downregulation as a result of irradiation. Using combined miRNA and mRNA expression profiles, we identified a set of miRNAs which may be crucial for the control of the radiation response some of which, like hsa-miR-34b, are directly controlled by p53 and other cell cycle-related transcription factors like...
MYC and E2F1. The relative contribution of different miRNAs to the regulation process also showed some cell line specificities which could explain variations in cell type-dependent differences in radiation response.

Conclusions: The results suggest a significant role of microRNAs in the processes of transcript level regulation in cells exposed to ionizing radiation. The miRNA levels and their changes after irradiation show cell-type specificities. The up-regulation of miRNA observed 12 h after irradiation seems to be mainly a consequence of a relaxation of effects of miRNAs which generally leads to an increased rate of transcript degradation. This work was supported by BK-274 Rau1/2011 and N 5518 497639.

[67] Modified Transcriptional Profile and Altered Cell Cycle, Survival and Oxidative Functions of Sox1-defective Fibroblasts

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Sos (Son of Sevenless) family proteins are Ras guanine nucleotide exchange factors (GEFs) playing a crucial role in coupling ligand-induced tyrosine kinase receptor (RTK) activation to Ras-mediated signaling pathways. Ras GTPases are important regulators of fundamental cellular processes such as gene expression, cell cycle progression, differentiation and survival. The overexpression of Sox1 isoforms of these family proteins has been reported in some types of tumors including prostate cancer. In this study we performed oligonucleotide microarray analysis to characterize the differential gene expression profile of mouse embryonic fibroblasts (MEFs) harboring homozygous null mutation in Sox1 locus (MEFs ko-Sox1). The functional relevance of the differentially expressed genes in absence of Sox1 was evaluated using bioinformatic tools. We also generated stable Sox1 knockdown cell lines by using shRNA strategy (shSox1 cells) to validate the functional alterations by means of proliferation and survival assays. We identified altered gene expression and a significant enrichment in a set of functional categories affecting several biological processes).

Results: The Sox1 knockdown cell lines by using shRNA strategy (shSox1 cells) to validate the functional alterations by means of proliferation and survival assays. We identified altered gene expression and a significant enrichment in a set of functional categories affecting several biological processes.

[68] Role of ATM Kinase in HER2-triggered Tumor Progression

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Background: HER2 is a receptor tyrosine kinase (RTK) that represents a key role in cell cycle progression, cell survival and stress response, among others. Moreover we found significant expression changes in some prostate cancer-related genes such as Gilr, Arnac and Eaf2. Finally, we observed defects in G1 phase progression, as well as increased reactive oxygen species (ROS) intracellular levels and mitochondrial membrane potential hyperpolarization in shSox1 cells compared with their controls.

Our data suggest that Sox1 is a positive regulator of cell cycle progression and survival. Its absence causes important gene expression changes which include some prostate cancer-related genes. We also unveiled a putative role of Sox1 in redox homeostasis by controlling ROS intracellular level and mitochondrial function.

Results: The Sox1 knockdown cell lines by using shRNA strategy (shSox1 cells) to validate the functional alterations by means of proliferation and survival assays. We identified altered gene expression and a significant enrichment in a set of functional categories affecting several biological processes.

[69] Aneuploidy Facilitates Oncogenic Transformation Via Specific Genetic Alterations

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Background: DNA content abnormalities of human cells have been reported in a large number of human tumors. The presence of aneuploidy is often associated with rapid growth and tumor progression, indicating a possible role of aneuploidy in oncogenic transformation. However, the mechanism underlying this process is still not fully understood.

Results: Gene expression profiling revealed that aneuploidy significantly altered the gene expression profile of these cells. Altogether, 157 genes were uniquely up- or down-regulated in the aneuploid MEFs when using a fold change threshold of 4. Among the many interesting hits of upregulated genes were Twist2, a transcription factor involved in induction of EMT and resistance to apoptosis. In addition, aCGH demonstrated that the chromosomal region of the Twist2 gene was located in a region of copy number gain on chromosome 1. Increased staining of Twist2 protein was also detected in the nuclei, which suggests increased activity as a transcription factor. Importantly, RNA-mediated silencing of Twist2 resulted in reduced invasion and anchorage-independent growth of the aneuploid cells. To further verify the involvement of Twist2 in malignancy, we also found elevated immunohistochemical staining of Twist2 protein in 3 out of 7 paraffin-embedded tissue sections of human sarcomas.

Conclusions: Together, these studies show that aneuploidy promotes a remarkable genetic evolution. In a context dependent manner, aneuploidy facilitates generation and selection of transformed cells that display specific alterations in gene expression and signaling. Inhibition of aneuploidy-induced pathways partially reverses cell malignancy.

[70] Phosphorylation of EIF4E Enhances Survival and Growth Under Stress Conditions

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Background: Regulation of protein translation is a central aspect infl uencing cancer development and progression. Eukaryotic initiation factor (eIF) 4E mediates the action of eIF4E. EIF4E associates with the mRNAs’ 5’ cap structure and is an essential factor for canonical protein translation. eIF4E acts as an oncogene when overexpressed in cell lines and high levels of eIF4E and phosphorylated eIF4E have been described in a large number of human tumors. EIF4E is also present in the nucleus and contributes to the transport of certain mRNAs from the nucleus to the cytoplasm. EIF4E has also been described as a component of cytoplasmic stress granules and processing bodies. We have previously shown that high levels of p-eIF4E were associated with resistance to radiation and chemotherapy in carcinoma cell lines. As the function of p-eIF4E is poorly understood, we evaluated the role of p-eIF4E phosphorylation in relation to proliferation and in vitro stress resistance.
Material and Methods: Cancer cell lines (MDA-MB 231, MBA-MB 468 and HeLa cells) and immortalized human keratinocytes (HaCaT) were infected with doxycycline-inducible hyperphosphorylated eIF4E-S209D (S209D) mutants of eIF4E. Cells were subjected to different types of stress including oxidative (Arsenite), nutrient starving and cisplatin treatment. Immediate and long term response to stress was evaluated using colony formation, MTT cell proliferation assays, co-immunoprecipitation and immunofluorescence microscopy.

Results: Significantly greater recovery after stress was observed in all the cell lines expressing hyperphosphorylated eIF4E-S209D. After nutrient starving or exposure to arsenite, we observed increased expression of several proteins containing AU-rich elements (AREs) in their 3’ mRNA region, including Cyclin D1, beta-catenin, 4E-T, McI-1 and Cx43. Intriguingly, this increase was specific for eIF4E-S209D and not eIF4E-S209A or GFP expressing cells. Under normal condition we observed a unique colocalization of 4E-T and eIF4E-S209D in cytoplasmic bodies. This complex colocalize with AgO2 but not with markers of the stress granules (TIA-1) and processing bodies (DCP1A). By co-immunoprecipitation, we observed that pS209E interacts with its carrier protein 4E-T, AgO2 and the ARE-binding protein Hur.

Conclusions: Phosphorylated eIF4E confer resistance to several stress conditions, including oxidative stress, DNA damage and nutrient deprivation. The fact that there was a relevant and significant increase of proteins such as Cyclin D1, McI-1 and beta-catenin, suggests that phosphorylated eIF4E plays an important role in selectively stimulating RNA translation under or immediately after stress.

Inhibition of eIF4E might enable the formation of a unique type of 4E-T/eIF4E cytoplasmic bodies.

Multiple Cytokine Expression in Colorectal Cancer

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Background: Colorectal cancers (CRC) is one of the major killers, with poor prognosis, reduced survival rates and unsatisfactory responses to therapy. Early diagnostics and improved disease characterization are major keys, while local and systemic modifications in relation with disease.

Material and Methods: Investigations were carried on serum samples collected from patient groups: colorectal cancer patients ~ (CRC group, n = 37, 15-82m, ages 44-82, clinical stages 2-4, control sera from healthy patients, n = 32, matched of the age and sex distributions. Serum cytokines were determined in sera by Luminex® 200 (Luminex Corp., TX, USA) using Milliplex MAP Human Cytokine/Chemokine Panel (Millipore, MA, US) according to manufacturer’s instructions. Multiplex data acquisition was performed using Starstation 2.3 (Applied Cytometry Systems, Sheffield, UK).

Results: Levels of expressions for GM-CSF, INF-g, IL-1β, IL-2, IFN-γ, IL6, IL7, IL8, IL10, IL12p70, IL-13, MCP-1 and TNF-α were determined in patients and control sera using the multiplex-xMAP technology. Modified expression levels were recorded for several cytokines; GM-CSF, INF-g, IL8 and IL-8 appeared overexpressed with ratios higher than 2 in all stages. For some of these cytokines, the levels of overexpression appeared to correlate with tumor stage. For IL-4, IL-5, MCP-1 and TNF-α a significant levels of overexpression (>2) were found only in stage 4 tumors, while in lesser stages the level of overexpression was lower.

Conclusions: Some pro-inflammatory and remodeling cytokines are overexpressed in CRC. They correlate well with stage, proliferation markers and clinical aggressiveness. The profile of over-expressed molecules associates with local and systemic modifications in relation with disease.

Akt Isoform-specific Signaling in Prostate Cancer

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Mutations and amplifications in genes regulating the PI 3-K/AKT pathway are common in prostate cancer, with inactivating mutations or deletions of PTEN being the most frequent. This has led to research on anti-cancer drugs targeting the Akt pathway. Although the role of Akt in tumorigenesis has been studied extensively, relatively little is known regarding isoform-specificity, and it is largely assumed that Akt isoforms play redundant roles in regulating cancer cell survival. In this study, we have developed a tet-on inducible system for expressing Akt1/2/3 shRNA to determine the role of Akt isoforms in prostate cancer progression. We have generated stable clones of the prostate cancer cell lines expressing active and PTEN-deficient prostate cancer cell line. Three-dimensional (3D) matrigel cultures are employed to better recapitulate tumors growing in vivo. Administration of doxycycline (dox) in both Akt1 and Akt2 shRNA LNCaP cells at the start of the assay results in inhibition of spheroid growth compared to control. Therefore, both Akt1 and Akt2 are necessary initially for growth in 3D cultures. Conversely, when spheroids were allowed to form for 7 days, followed by dox for further 10 days, Akt1-depleted spheroids were unchanged compared to control. In contrast, silencing of Akt2 resulted in a complete disintegration of spheroids at 17 days, with a noticeable change in morphology at as little as 4 days post dox addition. Confocal microscopy studies showed a significant induction of active caspase-3 and fragmented nuclei in Akt2 silenced spheroids. Moreover, whereas Akt1 is uniformly expressed, Akt2 is localized in the cytoplasm and/or on the plasma membrane. Importantly, in contrast to the profound differences observed between Akt1 and Akt2 in 3D cultures, the differences of effect of Akt isoforms on cell survival in conventional 2D culture are considerably more modest. Finally, the exclusive requirement of Akt2 for survival is also observed in PC3 prostate cancer cells, which are also PTEN-deficient. These results suggest that Akt2, but not Akt1, plays a critical role in modulating prostate cancer cell survival. Current efforts are aimed at identifying novel Akt isoform-specific substrates that contribute to prostate cancer progression using an unbiased, genome-wide screen. The results of these studies could point to new targets for anti-cancer therapeutics.

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Beta-catenin Confers Resistance to PI3K and AKT Inhibitors and Subverts FOXO3A to Promote Metastasis in Colon Cancer

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Wnt/beta-catenin and PI3K/AKT pathways play a central role in cancer. We describe the function of their common binding transcriptional effectors – beta-catenin and FOXO3A – in colon cancer proliferation. Their simultaneous nuclear accumulation and activation promotes cell scattering and metastasis, by regulating a defined set of target genes. Unexpectedly, the anti-tumour AKT inhibitor API-2 promotes nuclear FOXO3A accumulation and metastasis of cells with high nuclear beta-catenin. beta-catenin confers resistance to FOXO3a-mediated apoptosis induced by PI3K and AKT inhibitors in patient-derived primary cultures and in corresponding xenograft tumors in mice. This resistance is reverted by Wnt/beta-catenin signaling inhibitor XAV-939. FOXO3a does not behave as a tumor suppressor but rather as a metastasis inductor activated by PI3K or AKT inhibitor drugs when acting in concert with beta-catenin. We show that it is possible to evaluate b-catenin status and the response of patient-derived cells to these target-directed drugs before deciding on treatment. We also consider that this evaluation could be essential to the provision of a safer and more effective personalized treatment.

Mitochondrial Polymorphisms as Possible Predictive Markers in Human Papilloma Virus (HPV)-related Cervical Lesions

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Background: Cervical cancer is the second most common malignant neoplasm in women, in terms of incidence and mortality rates worldwide. High risk human papillomaviruses (hr-HPV) are known to be the etiological agents of cervical cancer disease. Developing new diagnostic tools that could be important in cervix carcinogenesis. Recently, mitochondrial DNA (mtDNA) have been found in many types of cancer. To investigate the possible role of single nucleotide polymorphisms (SNP) in cervix lesions we analyzed the D-loop sequence of patients with HPV-induced cervical lesions.

Material and Methods: In order to evaluate the role of T239C, A263G, C150T, T16189C and C16223T mtDNA polymorphisms in cervical lesions, cervical scrapings (from 60 HPV-cervical lesions) and 100 mtDNA isolated (High Pure PCR Template, Roche Diagnostics) from cervical tissues from patients with different cytology (normal cervical epithelium, ASCUS-Atyypical Squamous Cells of Undetermined Significance, LGSIL-Low-Grade
A Role for GPR55 in Multistage Mouse Skin Carcinogenesis

Role of CDX2 on the Regulation of the Cancer-associated Genetic Engineering of Oncolytic H-1 Parvovirus Capsid Increases STn−by concomitant positive regulation of carcinomas. We therefore hypothesized that CDX2 might induce a cancer-of MUC2 mucin expression, the major carrier of STn in IM and gastric However, the regulation of its expression is not fully elucidated. Previous to controls. We determined that the C150T and T239C polymorphisms were lower incidence of T16189C, C16223T in patients cervical lesions compared represented mainly by HPV16, 18, 31 and 33. We detected a significantly greater incidence of mdna polymorphisms T239C, A263G and C150T and a lower incidence of T16189C, C16223T in patients cervical lesions compared to controls. We determined that the C150T and T239C polymorphisms were strongly correlated with high risk HPV-positive LGSIL and HGSIL lesions (P < 0.05). Furthermore, HPV-positive individuals were more likely to carry the C150T, A263G and T16189C polymorphisms than HPV-negative controls (P < 0.003). A positive correlation between HPV-positive LGSIL patients and A263G SNP was found in our study group. In all SCC subjects, an increased risk of HPV infection was also associated with the selected polymorphisms (P < 0.02).

Conclusion: These findings suggest that mitochondrial SNPs in the D-loop region may represent a cofactor in HPV-induced cervical lesions and cancer but their role in the mechanism of carcinogenesis remains to be solved.

689 A Role for GPR55 in Multistage Mouse Skin Carcinogenesis

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Background: Increasing evidence show a link between the de-regulation of G protein-coupled receptors (GPCRs) and cancer. Recently, the orphan GPCR GPR55 has been proposed to modulate cancer cell proliferation and migration in vitro, and tumour growth in a xenograft-based model of glioblastoma. To further understand the role of GPR55 in cancer development, we studied the involvement of GPR55 in skin carcinogenesis in vivo.

Material and Method: We subjected GPR55 knock-out (KO) mice and their wild-type (wt) littermates to the two-stage model of skin carcinogenesis: animals were topically treated with a single dose of a carcinogen [7,12-dimethylbenz(a)anthracene (DMBA)], followed by repeated applications of the tumor promoter 12-O-tetradecanoylphorbol-13-aceta (TPA). This treatment results in the outgrowth of highly differentiated benign papillomas, which eventually progress to malignant squamous cell carcinomas (SCC). We also knocked-down the expression of GPR55 in a mouse skin carcinoma cell line, and analyzed oncogenic properties such as anchorage-independent growth, invasiveness and in vivo tumorigenesis. Finally, we analyzed the expression of GPR55 in human skin tumors and other squamous cell carcinomas by real-time quantitative PCR and by analysis of publicly available microarrays datasets.

Results and Discussion: Our results show that GPR55-deficient mice are significantly more resistant to DMBA/TPA-induced papilloma and carcinoma formation than their wt littermates. Different in vitro and in vivo approaches show that GPR55 confers oncogenic properties on cancer cells. Specifically, GPR55 increases (i) cancer cell proliferation (as indicated by the resistance of GPR55-deficient mice to TPA-induced epidermal hyperproliferation and by the co-localization of GPR55 with endogenous markers of proliferation), (ii) anchorage-independent growth and invasiveness in vitro, and (iii) tumorigenicity in vivo. Finally, GPR55 is upregulated both in human squamous cell carcinomas and mouse skin tumors compared to non-tumoral tissue.

Conclusion: Taken together, these findings suggest that GPR55 plays a pivotal role in skin tumour development, and that this receptor may be used as a new biomarker and therapeutic target in squamous cell carcinomas.

691 Role of CDX2 on the Regulation of the Cancer-associated Sialyl-Tn Carbohydrate Antigen


Background: De novo expression of Sialyl-Tn (STn) antigen is one of the most common features of gastric intestinal metaplasia (IM) and gastric cancer. However, the regulation of its expression is not fully elucidated. Previous studies identified the homebox transcription factor CDX2 as a direct regulator of MUC2 mucin expression, the major carrier of STn in IM and gastric carcinomas. Therefore, we hypothesized that CDX2 might induce a cancer-associated glycoproteome alteration in the gastric context – MUC2 carrying STn – by concomitant positive regulation of ST6GalNAc-I, which encodes the sialyltransferase responsible for STn biosynthesis, and MUC2 genes. In this study, our aim is to evaluate whether CDX2 transactivates ST6GalNAc-I gene in a gastrointestinal model, both in vitro and in vivo, and to identify CDX2 putative binding regions on this gene.

Material and Methods: To clarify the mechanisms underlying ST6GalNAc-I gene transcriptional regulation by CDX2, the transcript levels were assessed by qPCR in a model of induction and a model of silencing of CDX2, respectively: (1) during in vitro differentiation of Caco-2 intestinal cell line and 2) upon siRNA-mediated CDX2 silencing in the gastric cell line AGS. Co-transfection of gastric and colonic cell lines with pGL3-derived constructs covering 1.6 kb of the human ST6GalNAc-I promoter and a CDX2 expression vector was performed. Chromatin immunoprecipitation (ChIP) was carried out in intestinal Caco-2 cells in order to identify the relevant CDX2-binding regions to the ST6GalNAc-I promoter.

Results: In vitro spontaneous differentiation of Caco-2 cells induces an increase in CDX2 expression which is accompanied by concomitant increase in ST6GalNAc-I expression. On the other hand, CDX2 silencing using siRNAs in AGS cells was followed by a decrease in ST6GalNAc-I transcriptional levels. Luciferase assays confirmed a transactivation of ST6GalNAc-I promoter and ChIP analysis proved that CDX2 was bound to it.

Conclusions: Our work supports the novel concept that a single homeobox gene, CDX2, is orchestrating a glycoproteome modification during cancer development. Future perspectives include validation of the results by site-directed mutagenesis of the ST6GalNAc-I promoter, EMSA and the use of a Proximity Ligation approach for in vitro and in vivo detection of DNA-protein interactions. Moreover, in vivo ChIP will be performed using human gastric IM and cancer samples to validate our hypothesis on gastric carcinogenesis.

699 Genetic Engineering of Oncolytic H-1 Parvovirus Capsid Increases Virus Specificity for Cancer Cells

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Introduction: The rat H-1 parvovirus (H-1PV) is a promising anticancer agent because it is not pathogenic for humans and it is endowed with natural oncotropic and oncosuppressive properties. Although the virus preferentially replicates in tumor cells, it is also able to enter normal cells. The uptake of the virus by non-tumor cells is expected to diminish the therapeutic dose of the virus available for targeting the tumor, reducing its anti-neoplastic potential and raising some potential safety concerns. The aim of this study was to increase the virus specificity for cancer cells by genetically modifying the H-1PV capsid structure.

Materials and Methods: After silico modeling of H-1PV capsid, performed by homology with the resolved crystal structure of the closely related parvovirus minute virus of mice (MV), we searched for putative amino acids involved in cell membrane recognition. Then, with the aim of obtaining an entry-negative virion, we mutated these candidate residues and evaluated whether the mutation induced the virus affecting viral entry into patient cells. We then tested whether the insertion into the viral capsid of a cyclic RGD-4C peptide, which recognizes αvβ3 and αvβ5 integrins (often over-expressed in cancer cells and angiogenic vessels), may target the virus specifically to cancer cells.

Results: Similar to MV, H-1PV needs sialic acid as a component of the primary receptors required for viral entry, as the removal of sialic acid from the cell surface by neuraminidase treatment abolished viral infection into permissive cells. In situ mutagenesis of the amino acids G367 and H373, located on the 2-fold axis of symmetry, strongly reduced the ability of H-1PV to bind and enter into permissive cell lines. The insertion of the cyclic peptide RGD-4C at the level of the 3-fold axis of symmetry of the entry-deficient mutant virus H373R conferred to the virus a novel tropism specific for αvβ3 integrins, without impairing particle assembly, DNA replication and virus production. Coherently, the retargeted virus showed increased ability to infect and kill tumor cells overexpressing αvβ3 integrins while sparing their normal counterpart, in which αvβ3 integrins expression is low or absent.

Conclusion: This study showed that H-1PV capsid can be genetically modified in order to specifically redirect the virus to the surface of cancer cells while preserving its oncolytic potential.

700 Tumour Suppressor Activity of Cbx7 in Lung Carcinogenesis

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Background: The Cbx7 gene encodes a polycomb group protein whose role in carcinogenesis as a tumour suppressor or as an oncogene is still controversial. The loss of Cbx7 expression has been associated with increasing malignancy grade in different type of human neoplasias as
thyroid, colon, breast and bladder, whereas the retention of CBX7 expression correlates with a longer survival, thereby suggesting a tumour suppressor role. However, there is no evidence that CBX7 overexpression would lead to cellular immortalisation in vitro and tumour development in vivo, thereby proposing it as an oncogeno. To address this issue we generated and characterized mice null for the cbx7 gene.

Material and Methods: For the generation of knockout (KO) mice, we used gene targeting techniques in embryonic stem (ES) cells. Mouse tissues were analysed by standard histopathology. Growth curves and FACS analyses of cbx7−/− mice embryonic fibroblasts (MEFs) were performed to analyze growth properties. Cell cycle-related protein expression was analyzed by Western blot. EMSA, ChIP and luciferase assays were carried out to evaluate the role of CBX7 on the CCNE1 promoter. CBX7 and Cyclin E expression was analyzed by immunohistochemistry in human lung carcinomas and normal tissue.

Results and Discussion: Cbx7−/− mice develop liver and lung adenomas and carcinomas and the development of benign or malignant lesions correlates with the overexpression of cbx7−/− functioning alleles. Moreover, clear signs of lung epithelial cell hyperproliferation and dysplasia are present in young Cbx7−/− mice. Accordingly, cdx2−/− MEFs have a higher growth rate, with an increased number of cells in the S phase of the cell cycle, than their wild-type counterparts. This was associated with the expression of multiple cycle cell effectors, including cyclin E, which is known to play a key role in lung carcinogenesis. We showed that CBX7 is able to bind the CCNE1 promoter, in a complex that included histone deacetylase 2 (HDAC2), and negatively regulates Cyclin E expression. Consistently, cyclin E upregulation negatively regulates Cyclin E expression. Consistently, cyclin E upregulation was observed in Cdx2−/− tissues and MEFs with respect to the wild-type counterparts. Finally, we found that the lack of CBX7 expression in human lung carcinomas correlated with CCNE1 overexpression.

Conclusion: The generation of cbx7−/− mice validates a tumour suppressor role for CBX7, whose loss plays a relevant role in lung carcinogenesis.

Cadmium Stimulates Urokinase Plasminogen Activator Receptor Expression Via ROS and Erk-1/2 Pathway Through NF-kB and AP-1 Signaling in Human Gastric Cancer Cells

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Introduction: Cadmium (Cd) is a hazardous heavy metal that threatens to living organisms’ functions. The exposure to Cd can lead to apoptosis or carcinogenesis in human cells. It is well known that D can cause carcinogenesis in human, especially for lung cancer of cigarette smoking. However it is less known about the effects of Cd on the gastric cancer. The purpose of this study was to reveal the mechanisms by Cd stimulation in proliferation and metastasis potential for AGS cell line was elevated by crystal violet staining. MTT analysis, matrigel invasion assay, respectively. Northern blot and western blot were used to examine the expression of USP4 and p-HOER1/2. Luciferase assay and EMSA were used to examine the effect of Cd in activation of transcription factors AP-1 and NF-kB.

Results and Discussion: Here we show that Cd significantly elevated USP4 expression in both a dose- and time-dependent manner. The increased level of USP4 by Cd was through extracellular signal-regulated kinases (ERK)-1/2, p38, JN, N-terminal kinases (JNK) MAPK signalling pathways. The activation of Erk-1/2 by Cd was resulted from induction of reactive oxygen species (ROS). Antioxidant, N-acetyl-L-cysteine (NAC) studies revealed that the ROS production by Cd is an upstream signaling for the Erk-1/2 expression. Furthermore AGS cells treated with Cd showed that activation of transcription factors AP-1 and NF-kB. We have also found in this study that Cd can stimulate cell invasiveness through the overexpression of USP4.

Conclusion: These data suggest that Cd, as carcinogen, elevated USP4 expression via ROS and MAPK pathway through NF-kB and AP-1 signaling in human gastric cancer cells.

Mutation Screening of BRCA1 Gene in Bulgarian Breast Cancer Patients

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Background: Germ line mutations in BRCA1 breast cancer (BC) susceptible gene account for the majority of hereditary breast and ovarian cancers.

Material and Methods: We have screened 200 Bulgarian BC patients fulfilling the BCLC criteria for germ-line mutations in BRCA1 gene. The mutation screening was performed by direct sequencing and MLPA and the results were further analyzed by SeqScape and Cofalysfer software.

Results: Totally 28 BRCA1 mutation carriers were found (14%). The founder mutations described in Ashkenazi Jews were spread over all of Europe. In SBNCNSBRCA1 exon 20 was observed in 23 patients (11.5%), 13 of which demonstrated TNBC subtype. The frequency of the large genomic BRCA1 alterations was 2%, which was in consistency with previous studies in other populations. Four genomic deletions or inversions were found in the BRCA1 gene: del BRCA1 ex 11A in patient with breast/ovarian cancer; del BRCA1 ex 13 in a women with early onset and family history of BC; dup BRCA1 ex 14–15 and dup BRCA1 ex 20 in two patients with family history. In addition we found one unknown nonsense alteration in exon 11, codon 1037 (T>T), that leads to replacement of Val with Ala in patient with bilateral breast cancer developed by the age of 45.

Conclusions: The frequency of the BRCA1 mutations in the studied cohort of BC patients was 14%. Our results suggest that 5382insC might be the most frequent mutation (11.5%) among BC patients fulfilling the criteria for BRCA1 genetic testing in Bulgaria.

Comparison of Minor Groove Binding Ligands and Known Carcinogen Blastomogenic, Reombinogenic and Mutagenic Activity Revealed by SMART in Wts+/− Heterozygous Flies

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Background: Loss of heterozigocity (LOH) through homologous recombination, causing manifestation of a recessive mutant allele, has been shown to represent a frequent DNA rearrangement in tumors of patients with inherited relniblastoma and Li-Fraumeni syndromes. However it is less known about the effects of Cd on the gastric cancer. Results and Discussion: Here we show that Cd significantly elevated uPAR expression in both a dose- and time-dependent manner. The increased level of uPAR by Cd was through extracellular signal-regulated kinases (ERK)-1/2, p38, JN, N-terminal kinases (JNK) MAPK signalling pathways. The activation of Erk-1/2 by Cd was resulted from induction of reactive oxygen species (ROS). Antioxidant, N-acetyl-L-cysteine (NAC) studies revealed that the ROS production by Cd is an upstream signaling for the Erk-1/2 expression. Furthermore AGS cells treated with Cd showed that activation of transcription factors AP-1 and NF-kB. We have also found in this study that Cd can stimulate cell invasiveness through the overexpression of USP4.

Conclusion: These data suggest that Cd, as carcinogen, elevated uPAR expression via ROS and MAPK pathway through NF-kB and AP-1 signaling in human gastric cancer cells.

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Conclusion: These data suggest that Cd, as carcinogen, elevated uPAR expression via ROS and MAPK pathway through NF-kB and AP-1 signaling in human gastric cancer cells.
Role of Exosomes in the Regulation Tumour Progression

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Background: Extracellular membrane vesicles originally described as cargo for excessive proteins, are recognized as a powerful mechanism for the horizontal transfer of proteins and genetic information. According the current state of knowledge, there are several types of vesicles exist. However, only two types are characterized: exosomes, 40-100 nm diameter vesicles, derived from the intraluminal membranes of multivesicular bodies; and microvesicles, 200-1000 nm diameter vesicles which bud directly from the cell membrane upon different stimuli, and the apoptotic bodies produced by the cells undergoing apoptosis. Although the mechanisms of functions of exosomes and microvesicles are still poorly understood, their function in carcinogenesis is non-controversial. Tumour cells produce increased amounts of exosomes as compared to their non-transformed counterparts. We have previously demonstrated, that pancreas carcinomas, expressing the transmembrane protein tetraspinin 8 (Tspan8), produce exosomes which are able to induce angiogenesis, specifically recruiting endothelial cells and endothelial progenitors-like cells.

Materials and Methods: In our current research we have further investigated molecular mechanisms, by mean of which Tspan8 and Tspan8-containing exosomes contribute to lymph- and angiogenesis. For that a comparative proteome analysis of Tspan8-positive and Tspan8-negative colorectal cancer exosomes has been performed.

Results: We have defined several proteins, which were specifically enriched in the Tspan8-positive but not in the Tspan8-negative exosomes. Among them, the 67 kDa laminin-receptor and the Mac-2 binding protein, Mac-2BP were detected. Exosomes, enriched on these proteins interacted with them, the 67 kDa laminin-receptor and the Mac-2 binding protein, Mac-2BP were detected. Exosomes, enriched on these proteins interacted with endothelial and lymphatic endothelial cells, stimulating their proliferation and morphogenesis. Currently, we verify if Gal1 and Gal3 galexins, expressed on both types of the recipient cells and binding Mac2-BP are sufficient for the interaction between exosomes and cells.

Conclusions: These results highlight a new mechanism how cancer cells might influence their microenvironment stimulating angiogenesis and lymphangiogenesis. In vivo trials should verify, if Tspan8-positive exosomes are sufficient to induce angiogenesis and lymphangiogenesis in vivo.

Role of Rac1 in DEN-induced Liver Carcinogenesis

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Introduction: The best characterized members of the Ras-homologous (Rho) family of small GTPases are RhoA, Rac1 and Cdc42. They are known as function as a regulator of transcription and because of its nuclear localization, initiation and progression has not been reported. Based on its exceptional to date. Diethylnitrosamine (DEN) is a DNA-methylating agent and leads to the liver carcinogenesis model enabling liver-specific deletion of the Rac1 gene. 14 days old male Rac1¹⁻ and Rac1¹⁺ animals were treated with DEN (10 mg/kg; i.p.). Tumor growth was analysed after 40 weeks. Macroscopical visible tumors in liver and lung were counted and their size was measured. Neoplastic areas were analysed in HE-stained tissue sections.

Results: About 30% of the Rac1-wildtype animals (5 of 16 mice) had macroscopically visible tumors in liver. In contrast to tumors could be detected in Rac1-knockout animals (12 mice). We also found tumors in the lung, which was the case in 20% of the Rac1¹⁻ and in 40% of the Rac1¹⁺ mice. The results of microscopical analyses of HE-stained tissue sections will be presented and discussed.

Conclusion: Our findings show that Rac1 contributes to liver carcinogenesis following DEN treatment. Apparently, loss of Rac1 protects against tumor formation in the liver but promotes metastasis in the lung.

Decreased Mitochondrial Mutant Rate by Pulsed Magnetic Field Exposure

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Background: Aging is a multifactorial process influenced by different genes. Rapidly mutant cells cannot repair double strand breaks (DSB) via homologous recombination (HR). HDF1 is the yeast homolog of the Ku70 gene found in mammalian cells. Hdf1 protein forms a heterodimer with Ku80 homolog that binds with high affinity to DNA ends in DSB, resulting that nonhomologous end joining (NHEJ) pathway is involved in hdf1 mutants. In addition, the inactivation of these genes contributes to an accelerated aging but there is no report about metabolic alterations. White petite is a mitochondrial mutant in which mitochondria are not functional, so these cells can not get their energy through respiration. They must necessarily rely on the fermentation. It allows distinguishing them from wild strains, as they are unable to grow using glycerol as a carbon source. The index of respiratory competence (IRC) is the percentage of oxidative cells in relation to the total cells (oxidative + fermentative). The aim of this work is to study the role of RAD52 and HDF1 genes in the IRC during cellular aging.

Material and Methods: Cells and culture: Cells used were the yeast strain S. cerevisiae W38105−1C (genotype: MATaalpha, ade2, arg4−17, 1prl−289, ura3−52) (wt) and the rad52Δ and rad52Δ hdf1Δ derivatives. They were grown in rich medium (YPD). To study chronological aging, yeast cells were grown in a synthetic dextrose complete (SDC) medium and to test IRC, they were grown inYPD/Glyc broth.

Aging of cultures: SDC flasks were inoculated with 1,500,000 cells/ml and cultured (4 days) to reach early stationary phase. Then, the aging process started maintaining them at 30°C, 300 rpm, 20 days.

IRC: On days 0, 7, 14, and 20 of aging, cells were seeded on YPD and YPD/Glyc plates. Colonies grown were scored and IRC calculated as: IRC = (colonies on YPD/Glyc)/(Total colonies on YPD).

Results: A decrease in the values of IRC during chronological aging was observed in mutant cells in relation to wt. The inactivation of hdf1 and Rad52 genes produced a decrease in respiration during the aging process, reaching values of 30% and 70% at 20 day of aging, respectively, in relation to wt (100%).

Conclusion: The inactivation of Rad52 and mainly hdf1 leads to a decrease in the value of IRC during cellular aging. Therefore, the absence of DSB repair mechanisms (HR and mainly NHEJ) and chronological aging contribute to a metabolic state with high rates of fermentation.

Decreased Mitochondrial Mutant Rate by Pulsed Magnetic Field Exposure

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Background: Yeast mitochondrial mutants are cells in which mitochondria are not functional (white petites). By this reason these cells can not get their energy through respiration, so they need the fermentation machinery. These mutants are very useful as biological model in the study of phenomenon and factors that can alter the metabolism, like aging and exposure to pulsed magnetic field (PMF). Since more than twenty years ago, the study of magnetic field effects have been increasing but until know there is no reports published about clear effects, mechanisms of action, and/or dose-response relationships. The aim of this work is to study the evolution of the mitochondrial mutant rate that appears spontaneously by PMF exposure during the chronological aging of S. cerevisiae.

Material and Methods: S. cerevisiae cells (WS8105−1C) were cultured on YPD medium. They were exposed to PMF (25 Hz, 1.5 mT, 8 hours/day) during the aging process in SDC medium (40 days). During the aging period, the surviving fraction was measured by clonogenic assay. In addition, to test for mitochondrial mutants, yeast samples were seeded on YPD plates and colonies grown checked on a selective growth medium (YPGlic) by replica plating. Every 2 days of incubation, the number of mutants was measured in PMF-exposed and control unexposed samples.

Results: The results found show that the exposure to PMF induced a decrease in the total number of mitochondrial mutants grown (9 white petites/1000 survivors) in relation to the unexposed control samples (19 white petites/1000 survivors). Moreover, a different evolution of the number of mutants was observed during the aging phenomenon. White petites appeared spontaneously in control cultures increasing its number gradually during aging (from 10 to 100 white petites/1000 survivors), reaching higher values from day 20 to 40 of aging. However, samples exposed to PMF maintained the mutation rate constant at a value about 20 white petites/1000 survivors.

Conclusion: The exposure of yeast cultures (25 Hz, 1.5mT, 8 hours/day) during chronological aging decreases the mitochondrial mutant rate. In this way, the PMF applied could act as a protector agent against spontaneous mitochondrial mutations.
Escherichia Coli and Colorectal Carcinogenesis

Animal Model of the Papillary Thyroid Carcinoma Induced by Escherichia Coli E. coli is a consistent commensal of the human gut microbiota but some pathogenic strains have acquired the ability to produce toxins that can interfere with eukaryotic cell cycle or directly induce DNA damages. In this study, we analyzed the E. coli population associated with the colonic mucosa of human colorectal cancers and investigated the ability of CRC-associated E. coli to induce colonic tumorigenesis in multiple intestinal neoplasia (Min) mice with mutation in the APC gene, which is altered in the majority of CRC.

Material and Methods: Tumors and normal mucosa from CRC patients (n = 52) and healthy mucosa for diverticulosis controls (n = 30) were collected. Mucosa-associated and internalized E. coli were quantified and characterized. Min mice were infected with E. coli strain (strain CRC20, non pathogenic E. coli strain (strain K-12 MG1655) or PBS alone. Fecal bacterial colonization was quantified. Colonic samples were analyzed (macroscopy, histology).

Results: Significantly higher numbers of mucosa-adoherent and mucosa-internalized E. coli were observed in tumors compared to normal tissue from control and CRC patients (at distance of the tumor). A statistically significant relationship was found between the presence of mucosa-associated E. coli and poor prognostic factors for CRC as tumor staging, lymph node status. In vitro, CRC-associated E. coli strains were able to invade and to persist in intestinal epithelial cells. Electron microscopy analysis of infected cultured cells indicated that bacteria induce the elongation of eukaryotic cell membranes and are internalized within endocytic vacuoles. Infections of Min mice with CRC20 E. coli indicated that this strain was able to persist at a high level in the gut up to 10 weeks after infection in comparison to non pathogenic E. coli that was no longer detected 2 weeks after infection. CRC20-colonized mice showed a marked increased number of visible colonic polyps at 10 weeks compared to controls. Histological analyses confirmed that all these polyps were adenocarcinomas.

Conclusion: These data support that mucosa-adoherent pathogenic E. coli may be a cofactor in the pathogenesis of colorectal cancer.

Role of the Transcription Factor Forkhead Box P3 in Breast Cancer and Metastasis

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Background: The transcription factor forkhead box P3 (FOXP3) is implicated in the regulation of immune system development and function and it has been recognized recently, as expressed in tumor cells. We recently reported that FOXP3 expression in breast cancer was associated with worse overall survival probability and the risk increased with increasing FOXP3 immunostaining intensity. FOXP3 was also a strong prognostic factor for distant metastases-free survival, but not for local recurrence risk. GSEA analysis of a published dataset relative to MCF7 breast cancer cells expressing or not FOXP3, indicated that this transcription factor induces expression of several genes implicated in migration and metastasis. We investigated the involvement of FOXP3 in the metastatic process using in vitro and in vivo models.

Material and Methods: MDA-MB-231 breast cancer cell line, expressing low levels of FOXP3, was used to establish a let-off inducible expression system, in which full-length (WT) FOXP3 or splice variant form (Δ2) were induced upon doxycycline removal. The consequences of FOXP3 over-regulation were examined by proliferation, migration and invasion assays. Δ2 FOXP3-tet-off stable clone was injected subcutaneously in SCID mice with or without the administration of doxycycline. Tumor weight and the number of spontaneous lung metastases were compared between the two experimental groups.

Results: Real Time PCR and Western Blot analysis confirmed that FOXP3 mRNA and protein levels were significantly increased in both WT and Δ2 FOXP3-tet-off MDA-MB-231 cell lines upon doxycycline removal. Up-regulation of WT and Δ2 FOXP3 inhibited proliferation, increased migration and invasion in vitro.

In our in vivo model, tumor weight and volume in Doxi-taking mice were significantly larger than corresponding measurements in Doxi-free mice. At the end of the observation period, a high percentage of Doxi-free mice developed a local secondary tumor whereas no secondary tumor development was noted in the corresponding Doxi-taking group.

Furthermore, an increased number of lung metastases was observed in Doxi-free mice compared to Doxi-taking mice.

Conclusions: Metastasis is a process requiring the activation of several pathways, and a transcriptional factor such as FOXP3, able to regulate numerous genes, might be a relevant player. Our data suggest that FOXP3 expression in breast tumor cells inhibits tumor growth and promotes metastatic capability.

K-Ras and CDKN2A Mutations in Pancreatic Cancer

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Introduction: Pancreatic cancer is the most fatal of all gastro-intestinal malignancies with a poor median survival of less than 5%. The tumor heterogeneity, lack of prognostic markers for early detection, and the poor response to therapy make this disease difficult to treat. The K-RAS gene mutations are frequent and early events in the development of pancreatic adenocarcinoma and can be used as a diagnostic and prognostic tool.

Material and Methods: Mutations were screened in four important cancer associated genes K-RAS, CDKN2A, B-RAF and GNAS in 171 resected exocrine tumors with at least 10% tumor content. Point mutations were identified using single stranded conformation polymorphism (SSCP) in the codon 11, 12, 13 and 61 of K-RAS, exon 1 and 2 of CDKN2A, codon 201 of GNAS, and codon 600 of B-RAF genes. The mutations were confirmed by DNA sequencing. The deletions in CDKN2A locus were characterized using multiplex ligation-dependent probe amplification (MLPA) and analysed using Coffalyser software. All the experiments were repeated for reproducibility. Statistical analyses were done to determine proportional hazard ratios using SAS 9.2.

Results: Mutations in K-RAS were found in 134 tumors (78%) with 131 of those in codon 12 and only 3 in codon 61. The G12D mutation accounted to 61%, followed by G12R (18%) and G12V (17%). Alterations in CDKN2A were detected in 43 tumors; GNAS mutations were present in two tumors and none in B-RAF. Presence of mutations in K-RAS gene were associated with a reduced patient overall survival by half (17 vs 30 months, log-rank P = 0.07) with a multivariate hazard ratio (HR) of 1.87 (95% CI 0.99-3.51). The patients with G12D mutation in tumors showed a median overall survival of 16 months (log-rank P = 0.02; HR 1.99 (95%CI 1.02–3.90)). Though, the association of mutation in patients with CDKN2A alterations in tumors was not statistically significant, the sub-set of patients with concomitant K-RAS mutations and CDKN2A alterations in tumors showed poorest median overall survival (13 vs 30 months, log-rank P = 0.03) and corresponding HR 2.77 (95% CI 1.23–6.23).

Conclusions: Mutations in K-RAS are frequent but not universal in pancreatic tumors. The presence of specific G12D K-RAS mutation was associated with poor survival and could potentially be used as predictive marker. Concomitant occurrence of K-RAS mutations and aberrations in CDKN2A resulted in a subgroup of patients with lowest survival.

Animal Model of the Papillary Thyroid Carcinoma Induced by BRAFV600E Mutation

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Introduction: A significant increase in the frequency of BRAFV600E mutation in papillary thyroid carcinomas (PTC) and a decrease in the incidence of the second major genetic alteration in PTC, RET rearrangements was recently observed. Association of BRAF mutation with aggressive phenotype of PTC and poor prognosis strengthen the need of getting know better the molecular background of PTC induced by the BRAFV600E mutation. The aim of our study was to obtain the mouse model of the BRAFV600E induced papillary thyroid carcinoma for the analysis of BRAFV600E influence on the gene expression profile of PTC.

Material and Methods: Transgenic mice were obtained by injection of the mutated human BRAF gene (pMEV-2HA) plasmid, Bmiomyx) under the bovine thyroglobulin promoter (obtained by courtesy of Prof. J.E. Dumont) into the pronucleus of one-cell mouse embryos (FVB/N). After 6-12 months the
Stimulation of Angiogenesis by Hepatitis C Virus (HCV).

**Background:** Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide. HCC tumors are highly malignant and characterized by active neovascularization. One of the major risk factors for developing HCC is infection with hepatitis C virus (HCV). The precise molecular mechanisms that link HCV infection to the development and progression of liver cancer are not entirely understood, but increasing evidence indicates that stimulation of angiogenesis by HCV may contribute to HCC malignancy. However, the angiogenic activity of HCV has, so far, only been demonstrated in the chicken chorioallantoic membrane (CAM) assay.

**Material and Methods:** Conditioned medium (CM) of Huh7 hepatoma cells, Huh7 cells infected with HCV, or Huh7 cells stably expressing the HCV subgenomic replicon (containing the nonstructural proteins NS3-NS5) was collected. Different steps of the angiogenic cascade, including endothelial cell proliferation, motility, migration, invasion and tube formation in the presence of Huh7 CM were analyzed. PCR angiogenesis array, real-time RT-PCR, Western Blot and Elisa were performed to investigate the expression of angiogenic factors in the different Huh7 cell cultures. Electroporation was used to introduce individual viral genes into Huh7 cells or to inhibit expression of angiogenic factors by small-interfering RNAs.

**Results:** HCV did not affect endothelial cell proliferation. In contrast, endothelial cell migration, invasion and tube formation were significantly increased in the presence of CM of HCV-infected hepatocytes. Comparable results were obtained by expression of the HCV subgenomic replicon in endothelial cells and maintenance of pancreatic tumour cells.

**Conclusions:** Our study included analyses in mouse models of acinar to ductal metaplasia (ADM) and in PDAC patient-derived tissues and cell-lines. We performed a multi-approach assessment of Dbc1 and Sirt1 expression and of the function of Sirt1, the effector protein.

**Results and Discussion:** Dbc1 and Sirt1 are co-expressed in the nucleus of normal exocrine acinar cells. Expression is aberrant in ADM and PDAC, with Sirt1 undergoing a transient cytoplasmatic shuttling and Dbc1 showing reduced expression, respectively. Inhibition of Sirt1’s intracellular shuttling or activity restrains ADM. In concordance, overexpression of Sirt1 impinges on acinar cell differentiation. We also identified that Pit1a and b-Catenin are targets in the exocrine pancreas for Sirt1 mediated deacetylation. Consequently, abnormal expression of Sirt1 can impact on Pit1a transcriptional activity and b-Catenin/Wnt signalling, mechanisms important in acinar cell differentiation.

**Conclusion:** Our study is the first to illustrate that abnormal subcellular localisation of Sirt1 impacts on the premalignant changes in exocrine cell differentiation and that reduced Dbc1 expression levels affect Sirt1 targeted druggability of PDAC tumour cells. Together, these observations suggest that targeting Dbc1/Sirt1 may have therapeutic potential throughout the course of pancreatic carcinogenesis.
The Influence of WWOX Tumor Suppressor Gene on Colorectal Carcinogenesis – A Microarray Study on SW480 Colon Cancer Cell Line

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Background: Despite remarkable progress in understanding the molecular process of colorectal tumorigenesis, this type of cancer is still one of the leading cause of tumor associated deaths in western countries. There are three major molecular pathways of colon carcinogenesis. The majorities of cases are characterized by chromosomal instability and CpG Island Methylator Phenotype and acquisition of Microsatellite instability are representing the other two pathways. Cancers arising from particular pathway have different allelotype at some of the loci. SW480 cells transduced with were numerous oncogenes and tumor suppressor genes involved in colon cancer initiation and progression including those associated with epithelial-mesenchymal transition. One of recently recognized is a tumor suppressor gene WWOX from the common fragile site FRA16D which has been shown to be associated with carcinogenesis of colon, breast and many other tumor types. WWOX integrates with several transcription factors and seems to play a role in controlling variety of molecular processes (cell differentiation, signal transduction, apoptosis, proliferation).

Material and Methods: WWOX cDNA was introduced into SW480 colon cancer cell line by retroviral transduction.

Using Real-time RT-PCR we estimated relevant expression of 10 genes regulating the basic cell processes (i.e. apoptosis, proliferation, adhesion and cell cycle (T.PAX, BCL2, KI67, BIRC5, CDH1). Global gene expression comparison between cells with elevated and native WWOX transcription was performed using DNA microarrays (Human OneArray™; Phalanx Biotech; 30 000 oligonucleotides probes). Biological tests of growth in soft agar, invasion, viability, proliferation and apoptosis assays were also performed to determine SW480 cells phenotype changes.

Results: Microarray analysis revealed over 900 differentially expressed genes in consequence of elevated WWOX expression (p<0.05). Our study demonstrated that WWOX has impact on variety of cellular pathways (e.g. downregulate of WNT, integrin and PDGF, TGf-signal pathway and upregulation of apoptosis, p53 pathway). Moreover, we found that genes regulating developmental processes are overexpressed in presence of increased WWOX protein level in contrast to some of genes regulating cell cycle which were downregulated. SW480 cells transduced with WWOX cDNA were characterized by lowered proliferation and viability but higher apoptosis. We have also noticed complete inhibition of cell growth in soft agar and reduced invasion of SW480 cells harbouring WWOX cDNA.

Conclusions: Our data emphasize the novel role of WWOX in modulation of particular pathways important in process of colon tumor development. As we assumed it has major impact on apoptosis, cell cycle and WNT pathway.

WWOX Modulates Gene Expression Profile in Glioblastoma T98G Cell Line

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Background: WWOX gene is localized in a common fragile site FRA16D. It is known to be a tumor suppressor. Unlike most of suppressor genes, loss of only one of its alleles is sufficient to alter its function in carcinogenesis – the haplosufficiency phenomenon. Although interactions with several transcription factors and signal transduction proteins (e.g. AP2, YAP, RUNX2) are well documented, it seems that we still know only a tiny piece of WWOX cellular role and its implications for carcinogenesis. Our previous experiments on glioblastoma tumor samples showed that WWOX expression level is correlated with other genes important to tumor formation and progression like Ki67, ErbB4, Bcl2. The current study was aimed to assess the influence of WWOX up-regulation on transcriptome of T98G glioblastoma cell line.

Material and Methods: WWOX cDNA was introduced into T98G glioblastoma cells by retroviral transduction. We used high-density microarrays – Human OneArraysTM (Phalanx Biotech) to study gene expression alterations in stable transductant cells having elevated WWOX expression in comparison with native (transduced with empty vector) T98G cells. The data analysis was performed with MeV (MultiExperiment Viewer) from TM4 package. For the ontological classification of genes we used Panther Classification System, which allowed determining which pathways are susceptible to change depending on WWOX expression level.

Results: The experiment revealed that increase of WWOX expression caused statistically significant changes in transcription of more than 3000 of other genes. Interestingly, most differentially expressed genes have shown rather induction than an inhibition of transcription in result of increased WWOX protein level. Expression changes induced by WWOX occurred among genes belonging to several essential signaling pathways: Notch (11 genes), EGFR (24 genes), PI3K (21 genes), Hedgehog (4 genes), WNT (48 genes), PDGF (26 genes). In the group of genes which expression was inhibited (p<0.05) we found that we can also find genes involved in metabolic processes, development, and intracellular transport.

Conclusion: The results of the experiment suggest that WWOX has a great impact on the expression of number of key genes in glioblastoma cell line T98G. WWOX triggered changes in expression of genes involved in cancer initiation and tumor progression in brain carcinogenesis.

Determination of Gene Expression in Endometrial Cancer Using Real-time RT-PCR

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Background: Endometrial cancer is the most common gynecological tumor in Poland and its morbidity rate is still increasing. For over 20 years scientists and clinicians adopted a dualistic model of endometrial carcinogenesis, showing significant differences in both molecular types of cancer. However, knowledge of the molecular biology of endometrial cancer is based primarily on a studies of individual gene expression changes, which do not show a comprehensive picture of cancer. Molecular pathology has very little diagnostic use and in practice diagnosis and prognosis is based on classical pathological report (FIGO clinical stage, myometrium invasion, invasion to the cervix, lymph node status, histological type and tumor size). Therefore it is important to study the endometrial carcinogenesis in a complex and synthetic view of its molecular biology based on parallel analysis of many genes involved in various cellular processes such as cell cycle regulation, proliferation, adhesion, apoptosis, signaling pathways and hormonal regulation.

Material and Methods: For the study we have used 143 samples of endometrial cancer and 28 samples of healthy endometrium.

Using a real-time RT-PCR method we have analyzed expression level of genes involved in process of proliferation (Ki67), apoptosis (Bax, Bcl2), signal transduction (EGFR, cell cycle (CCND1), cell adhesion (CDH1), transcription regulation (ESR1, NCOR1) and cytoskeleton construction (CK5, CK17), tumor suppressor gene (WWOX) using real-time RT-PCR. Moreover, we evaluated association of expression of studied genes with clinicopathological features (age, lymph node status, myometrium invasion, stage, grade).

Results: Statistical analysis showed a significant positive correlation between antipriopioptin index B2/Bax and expression level of tumor suppressor WWOX gene (R=0.39; p<0.0001) and Bcl2 (R=0.49; p<0.0001) but negative correlation with Bax (R= -0.69; p<0.0001), KI67 (R= -0.34; p<0.0001) and CK5 (R= -0.25; p=0.002). Analysis of clinicopathological prognostic factors revealed a decrease in expression level of Bax gene in poorly differentiated tumors (grade 3) compared with grade 2. Furthermore, expression level of WWOX tumor suppressor gene revealed tendency to decrease during transformation from normal to cancerous tissue.

Conclusion: These results suggest that imbalance in apoptosis process is crucial during endometrial carcinogenesis. Moreover, it seems that the WWOX gene demonstrates important role in the transition from normal to cancerous cells in human endometrium.

Snail Bypasses Senescence and Accelerates Tumor Progression in a Kras-driven Mouse Model of Pancreatic Cancer

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Background: Pancreatic ductal adenocarcinoma (PDAC) is a dismal disease with 5 year survival rates below 3 percent. The transcription factor Snail plays a crucial role in epithelial-mesenchymal transition (EMT) and is overexpressed in PDAC. We investigated the role of Snail for pancreatic carcinogenesis, metastasis and EMT in a genetically engineered mouse model of PDAC.

Material and Methods: We generated a latent Snail allele silenced by a lox-stop-lox (LSL) cassette as a knock-in at the murine Rasα26 locus (LSL-R26lox/lox). Expression of Snail in the pancreas can be activated in this model using a pancreas specific Cre driver line (Ptf1a Cre driver line). To investigate the role of Snail during pancreatic carcinogenesis, the LSL-R26lox/+ line was crossed into the established KrasG12D dependent PDAC model (Ptf1a Cre driver line). Phenotypic evaluation was performed by microscopic and microscopic pathological examination and molecular biology methods. Kaplan–Meier survival curves were used to compare the different compound mutant mice.

Results: Mice with pancreatic specific Snail expression from one Rasα26 allele exhibited normal phenotype. Increasing expression dose by expressing Snail from both Rasα26 alleles (Ptf1a Cre driver line) resulted in dramatic growth retardation, dedifferentiation of acinar cells and development of acinar cell carcinoma. In the context of concomitant
Elevated KitE Nin Can Promote the Progression of Colon Adenoma Within an APC Loss-associated Tumor Microenvironment

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Background: KitE Nin (KAI1 C-terminal interacting tetraspanin, Vangl1) is a novel tumor suppressor gene that might be relevant for colorectal cancer development. In this study, we investigated the role of KitE Nin in the progression of adenoma and colorectal cancer.

Results: We demonstrated that KitE Nin expression is significantly lower in colorectal adenoma and cancer samples compared to normal mucosa. Furthermore, forced expression of KitE Nin in colorectal cancer cells induced cell cycle arrest and apoptosis, while knockdown of KitE Nin expression promoted cell proliferation.

Conclusions: Our findings support the potential role of KitE Nin as a tumor suppressor gene in the development and progression of colorectal cancer.

Cyclooxygenase-2 Inhibition Enhances the Anti-tumour Activity of the Multi-target Kinase Inhibitor AEET788 in Colorectal Cancer Cells

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Introduction: Cyclooxygenase-2 (COX-2) is a key enzyme in the prostaglandin biosynthesis pathway and is overexpressed in various cancers, including colorectal cancer. AEET788 is a multi-target kinase inhibitor that has shown promising anti-tumor activity in preclinical studies.

Material and Method: We investigated the effect of AEET788 on COX-2 expression and activity in colorectal cancer cell lines. We also examined the potential of combining AEET788 with specific COX-2 inhibitors.

Results: AEET788 effectively inhibited COX-2 phosphorylation and activity, leading to enhanced apoptosis and cell cycle arrest in colorectal cancer cells. The combination of AEET788 and COX-2 inhibitors further potentiated these effects.

Conclusions: Our findings suggest that AEET788 may have therapeutic potential in colorectal cancer treatment through its anti-COX-2 activity.

Helicobacter pylori and the BMP Pathway in Gastric Cancer

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Introduction: Helicobacter pylori infection is a major risk factor for gastrointestinal malignancies. The BMP pathway is involved in the regulation of epithelial-mesenchymal transition and gastric cancer development.

Material and Method: We studied the role of BMP pathway modulation by H. pylori infection in gastric cancer cell lines.

Results: Our findings revealed that H. pylori infection induces BMP pathway activation, which contributes to gastric cancer cell proliferation and invasion.

Conclusions: Our results suggest that targeting the BMP pathway may be a potential therapeutic strategy for gastric cancer treatment.
validated and correlated with clinical and epidemiological data available for the study.

Conclusion: This multi-marker approach identifies a set of different markers that can be assessed in plasma, and that together (i) may be useful to narrow down the subjects at high risk of development of HCC among chronic liver disease patients in populations where chronic HBV carriage is endemic and (ii) represent very promising tools for improved, low-cost detection and diagnosis of HCC in high incidence, low resource regions of the world.

[77] Chemical Carcinogenesis in Mice With a Defective Epidermal Barrier – Exploring the Connection Between Skin Barrier and Cancer Susceptibility

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Introduction: The cornified envelope (CE) is the outermost layer of the skin and acts as the first line of defense that prevents from the invasion of pathogens and antigens from the outside and minimizes the water loss form the inside. Involucrin, envoplakin, and periplakin create the essential protein scaffold on which the envelope assemble.

Materials and Methods: In five- and three-gene (tKO) show a defective epidermal barrier and hyperkeratosis, resulting from impaired desquamation as compensation. In addition, tKO mice display a reduction in gd+ dendritic epidermal T cells and hyperkeratosis, resulting from impaired desquamation as compensation.

Results and Discussion: tKO mice are resistant to benign tumor development, showing reduced incidence and lower papillomas burden, compared with the relative wt controls. On the other hand, tKO mice show an increased malignant conversion from benign papillomas into malignant squamous cell carcinomas. Painting with DMBA induces the same levels of apoptosis, DNA breaks and proliferation in tKO and wt mice, claiming that the decreased tumour formation is not caused by defects in initiation. Promotion with TPA induces an exacerbated keratinocytes response (parakeratosis, hyperkeratosis, spongiosis and defective differentiation) and increased inflammation mainly made of CD3+CD4+ T lymphocytes with a Th2/Th17 profile, neutrophils and eosinophils in tKO mice, associated with increased levels of TSLP and IgE in the serum. This phenotype resembles what is observed in dermatitis patients.

Conclusion: Mice ko for envoplakin, periplakin and involucrin represent a useful model of skin barrier defect with features similar to individuals with atopic dermatitis. tKO mice are resistant to benign tumor formation, but display the same number of malignant tumors in a two stage skin carcinogenesis experiment compared with the relative wt mice. The exacerbated keratinocytes and inflammatory responses observed after promoting the skin with TPA are responsible for the decreased tumour formation. The mechanisms governing the higher malignant conversion in the triple ko mice are now under investigation.

[78] Combined Exposure to Multiple Carcinogens Enhances Development of Rat Mammary Cancers With Characteristic Gene Expression

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Introduction: Although mixed exposure to multiple carcinogens is common in human environment, the mechanism of cooperative carcinogenesis by two or more agents is poorly understood. In the present study, we undertook experiments using the rat mammary carcinogenesis model to assess the synergistic effect of combined exposure to γ rays and chemical carcinogens and potential underlying mechanisms.

Materials and Methods: Female Sprague-Dawley rats at 7 weeks of age were irradiated with γ rays (0, 0.5, 1 or 2 Gy); three days after irradiation, rats were subjected to single i.p. injection with 1-methyl-1-nitrosourea (MNU; 20 or 40 mg/kg) or 10 oral administrations with 2-aminomethyl-6-phenylimidazo[4,5-b]pyridine (PhIP; 40 mg/kg over two weeks), or left without chemical treatment. Tumors were examined pathologically after autopsy at 50 weeks of age. H-ras gene mutation was assessed by a restriction fragment length assay. Microarrays were used to search for genes characteristically expressed in carcinomas induced by combined exposures, followed by quantitative RT-PCR analysis incorporating additional tumours.

Results: Incidence of mammary carcinoma increased linearly as a function of radiation dose in the absence of chemicals. Administration of chemical carcinogens resulted in additive increase in cancer incidence. Significantly high (78%) prevalence of H-ras mutation was observed among carcinomas of rats exposed to γ rays (1 Gy) plus MNU (40 mg/kg) as compared to rats exposed to either γ rays (0%) or MNU (54%) only. Microarray and quantitative RT-PCR analyses identified characteristic gene expressions in carcinomas induced by combined exposures as compared to those induced by single carcinogens. For example, high expression of serum/glucoctocorticoid-regulated kinase (Sgk) was associated with carcinomas induced by γ rays (1 Gy) plus MNU (40 mg/kg) harboring H-ras mutation, whereas matrix metalloproteinase 17 was highly expressed in those without the mutation. Sgk may be induced synergistically by radiotherapy effects (e.g., oxidative stress, nitric oxide and TGF-β) and H-ras mutation and enhance proliferation of cancer cells. Carcinomas induced by γ rays (1 Gy) plus PhIP contained abundant transcription of the transcription factor Lef1, which may activate Wnt-responsive genes and stimulate cancer progression.

Conclusion: Interaction of oncogenic mechanisms at the molecular level may underlie the combined effect of multiple carcinogens on rat mammary carcinogenesis.

[79] Tumor Suppressors and Tissue Specificity in Cancer – the Role of Rad51c in Sebaceous and Mammary Carcinomas

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Background: Mutations in tumor suppressors are known to predispose to cancer. Less known is why these cancers show a distinct preference for a particular set of target tissues. Rad51c, a major protein of Homologous Recombination (HR), has been recently identified as a breast and ovarian cancer predisposition gene in humans and Kuznetsov et al. previously described Rad51c as a tumor suppressor with a tissue-specific phenotype in mice. Here, the loss of Rad51c in a p53-deficient background resulted in a strong prevalence of sebaceous carcinomas of preputial glands in males and mammary gland carcinomas in females, while the typical p53-related sarcomas were suppressed. The high incidence of preputial and mammary gland carcinomas seems therefore to be directly triggered by loss of Rad51c, making it a perfect animal model to investigate the mechanisms of tissue-specific tumorigenesis, with a special attention to the role of HR in cancer.

Materials and Methods: Organs and tumors from skin-specific Rad51c, p53 and Brca2 knock-out mouse cohorts have been analyzed immunohistologically and gene expression profiling is being used to identify the events associated with the tissue-specific loss of Rad51c.

Results: The loss of Rad51c is severely affecting preputial glands, causing depletion of mature sebaceous follicles, ectopic keratinization, increased apoptosis and impaired proliferation. Interestingly, loss of Brca2 does not lead to a similar phenotype, suggesting that Rad51c could have HR independent functions. Keeping in mind the findings from preputial glands, a similar approach was used to investigate the role of Rad51c in all developmental stages of the mammary gland. Preliminary data suggests that the loss of Rad51c alone in the mammary gland epithelium does not prevent the proliferation of the tissue, but further investigation is required. We have already collected several tumors and found a predominant sebaceous and squamous morphology. Currently, we are comparing data from the different cohorts and organs to pinpoint the events leading to tumorigenesis after Rad51c loss.

Conclusions: Rad51c is, together with two other tumor suppressors playing a critical role in Homologous Recombination (HR), namely BRCA1 and BRCA2, a breast cancer predisposition gene. Thus, understanding the mechanisms of its role in tumorigenesis at the tissue level will shed light on a broader role of HR in cancer and provide the translational link to human patients harboring germline mutations in Rad51c.

[80] Influence of Dietary Lipids on the Hepatic Xenobiotic Metabolism and its Importance on the Experimental Breast Cancer Initiation

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Background: Breast cancer is the most frequent cancer and one of the most important death causes in women worldwide. Diet, especially lipids, has an important role in its aetiology. In this study we have investigated the influence of a high corn oil (CO) and a high virgin olive oil (VOO) diets on...
the liver expression of phase I and phase II xenobiotic-metabolizing enzymes in an experimental breast cancer model. This influence could be a possible mechanism of the modulatory effects of dietary lipids on the breast cancer initiation.

**Materials and Methods:** Female Sprague-Dawley rats were fed two high fat diets (VOO and CO) or a control low fat diet from the weaning onwards. Breast cancer was induced by 7,12-dimethylbenz(a)anthracene administration at 53 days of age. Several kinds of hepatic protein and mRNA extracts were carried out and used to analyze the expression of diverse xenobiotic detoxification enzymes and that of their regulatory transcription factors by RT-PCR and Western Blot. Co-immunoprecipitation and activity assays were also performed.

**Results and Discussion:** The results showed reduced mRNA expression and protein abundance of the phase I enzymes (CYP1A1, CYP1A2 and CYP1B1) all through the time. This reduction correlated with the mRNA expression of the AhR transcription factor and its cytoplasmic retention by Hsp90. Moreover, diet seemed to affect the regulation of both detoxification phases. Thus, the VOO diet tended to decrease the mRNA and protein levels of phase I enzymes, except in the case of CYP1B1 protein. In contrast, phase II enzymes seemed to present an increased expression, but in a less clear manner. The CO diet exhibited an opposite effect on the hepatic expression of xenobiotic-metabolizing enzymes. Thus, the VOO diet would associate with a good balance between phase I and II enzymes in such a way that it would promote a more effective modification of xenobiotic compounds by phase I enzymes and their inactivation by phase II enzymes. This effect would avoid the escape of reactive metabolites to susceptible tissues as the mammary gland, where they could exert a genotoxic effect.

**Conclusion:** Our results drive us to assume that dietary lipids differentially affect the regulation of hepatic xenobiotic-metabolizing enzymes. This effect is in accordance with the previously demonstrated stimulating action of the CO diet and the potentially chemopreventive action of the VOO diet on breast carcinogenesis.

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**Identification of MYLK4 Mutations in Breast Cancer Cells and Analysis of Expression in Normal Tissues**

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**Background:** The gene MYLK4 (Mycos In Light Chain Kinase member 4) is located on chromosome 6, occupies an area of approximately 88 kbp and has a high expression level in breast cancer cells lines. These genes belong to the Protein tyrosine kinase 4 subfamily, which is involved in cell proliferation and apoptosis. MYLK4 has a high ‘CaMP score’ and is mutated in a panel of normal breast cell lines. The expression analysis was performed in 8 breast cancer cell lines and 12 normal tissues.

**Materials and Methods:** We selected 8 breast cancer cell lines (MCF7, CAMA1, SKBR3, MDA-MB-231, MDA-MB-436, MDA-MB-468, HCC2218 and HCC1954) and a matching cell line from normal breast tissues. The expression profile of MYLK4 in the breast cancer cell lines and in 12 normal tissues was studied.

**Results:** The expression analysis was performed using standard PCR technique. The M13 tag was attached to reverse primers to facilitate sequencing of the amplicons. The sequencing was performed using an ABI3700 DNA sequencer (Applied Biosystems®). The expression analysis was performed in 8 breast cancer cell lines and in a panel of normal breast tissues. Total RNA was isolated and cDNAs were synthesized. Quantification was performed using semi-quantitative PCR using a set of primers for MYLK4. All PCRs were normalized with β-actin.

**Conclusion:** Our results drive us to assume that dietary lipids differentially affect the regulation of hepatic xenobiotic-metabolizing enzymes. This effect is in accordance with the previously demonstrated stimulating action of the CO diet and the potentially chemopreventive action of the VOO diet on breast carcinogenesis.

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**DNA Repair Capacity in Peripheral Blood Lymphocytes of Endometrial Cancer Patients with Family History of Cancer**

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**Background:** It is well known that one of the causes of genetic instability is DNA repair deficiency. As a result cells become more sensitive to exogenous and endogenous DNA damaging agents leading to accumulation of mutations and increased risk of malignant transformation. Low efficiency in function of DNA repair genes may be consequence of inherited mutations of DNA damage-repair genes resulting in hypersensitivity to genotoxic substances. The aim of this study was to elucidate the role of DNA repair capacity in peripheral blood lymphocytes (PBL) of endometrial cancer (EC) patients considering family history of cancer.

**Materials and Methods:** Total 45 EC patients with the mean age 60.0 years and 10 healthy volunteers with the mean age 55.7 years were included in the study. PBL were exposed to bleomycin for 30 min (Schmezer et al., 2001). DNA repair capacity was assessed after 15 min incubation in RPMI 1640 without bleomycin. Assessment of basal and induced DNA damage in PBL was performed by DNA comet assay. DNA damage and DNA repair capacity were quantified as the mean percentage of DNA in the tail and the comet tail moment (TM).

**Results:** DNA repair capacity in peripheral blood lymphocytes of EC patients was significantly lower compared to PBL in healthy volunteers (TM 0.23 ± 0.11). The difference between the basal tail moments of PBL in endometrial cancer patients with family history of cancer didn’t alter significantly from patients with sporadic EC. Further when we conducted bleomycin treatment, there was an increase in the TM for PBL in endometrial cancer patients (99.62 ± 0.54) as well as in healthy volunteers (87.31 ± 0.99). Among the endometrial cancer patients the sensitivity of PBL to bleomycin was higher in patients with family history of cancer (TM 104.95 ± 1.16) in comparison with patients without family history of cancer (TM 97.02 ± 0.62). Following 15 min DNA repair after treatment with bleomycin the number of non-repaired DNA strand breaks in PBL has been more than 40% (DNA in comet tail 41.91 ± 0.98%) in endometrial cancer patients whereas in healthy volunteers its number decreased to 20% (DNA in comet tail 2.43 ± 0.11%). It has been revealed that the efficiency of DNA repair depended on family history of cancer in EC patients. After 15-min repair, PBL restored the damaged DNA to the level of TM 55.00 ± 2.43 in EC patients with family history of cancer and up to 22.77 ± 1.13 in patients without family history of cancer.

**Conclusion:** Peripheral blood lymphocytes of EC patients are characterized by genetic instability manifesting in the increased level of basal DNA damage, hypersensitivity to mutagenic factors and deficient DNA repair. In lymphocytes of EC patients with family history of cancer the hidden genetic instability is caused by increased susceptibility to mutagens and low DNA repair capacity is occurred.

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**Prolactin Promotes Hepatocellular Carcinoma Through Janus Kinase 2**

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**Background:** Hepatocellular carcinoma (HCC) is one human cancer with obvious gender disparity. This study investigated the association of aberrant prolactin levels with HCC risk and the potential impacts on HCC of the prolactin receptor (PRLR)/Janus kinase 2 (JAK2) signaling. In a prospective cohort study, the mutation analysis and correlation of the identified mutations to a possible functional change in ordertoestablishitsroleinthepathologyofbreastcarcinogenesis.

**Materials and Methods:** Serum prolactin of 63 HCC patients and 162 subjects without HCC was measured by radioimmunoassay. The expressions of PRLR and phosphorylated JAK2 (p-JAK2) in 82 retrospectively collected HCC patients considering family history of cancer. The Kaplan–Meier survival curve showed that high p-JAK2 expression was correlated with increased risk of malignant transformation. Low efficiency in function of DNA repair genes may be consequence of inherited mutations of DNA damage-repair genes resulting in hypersensitivity to genotoxic substances. The aim of this study was to elucidate the role of DNA repair capacity in peripheral blood lymphocytes (PBL) of endometrial cancer (EC) patients considering family history of cancer.

**Results:** DNA repair capacity in peripheral blood lymphocytes of EC patients was significantly lower compared to PBL in healthy volunteers (TM 0.23 ± 0.11). The difference between the basal tail moments of PBL in endometrial cancer patients with family history of cancer didn’t alter significantly from patients with sporadic EC. Further when we conducted bleomycin treatment, there was an increase in the TM for PBL in endometrial cancer patients (99.62 ± 0.54) as well as in healthy volunteers (87.31 ± 0.99). Among the endometrial cancer patients the sensitivity of PBL to bleomycin was higher in patients with family history of cancer (TM 104.95 ± 1.16) in comparison with patients without family history of cancer (TM 97.02 ± 0.62). Following 15 min DNA repair after treatment with bleomycin the number of non-repaired DNA strand breaks in PBL has been more than 40% (DNA in comet tail 41.91 ± 0.98%) in endometrial cancer patients whereas in healthy volunteers its number decreased to 20% (DNA in comet tail 2.43 ± 0.11%). It has been revealed that the efficiency of DNA repair depended on family history of cancer in EC patients. After 15-min repair, PBL restored the damaged DNA to the level of TM 55.00 ± 2.43 in EC patients with family history of cancer and up to 22.77 ± 1.13 in patients without family history of cancer.

**Conclusion:** Peripheral blood lymphocytes of EC patients are characterized by genetic instability manifesting in the increased level of basal DNA damage, hypersensitivity to mutagenic factors and deficient DNA repair. In lymphocytes of EC patients with family history of cancer the hidden genetic instability is caused by increased susceptibility to mutagens and low DNA repair capacity is occurred.
both p-JAK2 and cyclin D1 in Hep-G2 cells. Importantly, the proliferative effects induced by prolactin could be effectively attenuated by adding AG490, a JAK2 inhibitor.

Conclusions: Increased circulating prolactin was found in HCC patients and high p-JAK2 expression could predict poor overall survival in those patients expressing high levels of prolactin. In addition, prolactin contributed to the proliferation of liver cancer cells through PRLR/JAK2 signaling.

[724] Claspin Mutations and Loss of Function May Contribute to Breast Carcinogenesis and Gliomagenesis
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Background: Hereditary breast cancer is associated with germ-line mutations in BRCA1/2. However, these have incomplete penetrance, suggesting involvement of other factors. Being a BRCA1/2-interacting protein, Claspin may modify the risk of breast cancer development in BRCA1/2 mutation carriers or in familial cases in which no mutations in BRCA1/2 are identified. Claspin seems to have a central role in checkpoint control, namely through monitoring of DNA replication and bridging checkpoint responses to the DNA repair machinery, by interaction with different proteins, including BRCA1. Despite the multiple functions of Claspin in the maintenance of cell homeostasis, little is known about its role in cancer. Two recent studies have highlighted the importance of Claspin inactivation during human carcinogenesis, namely that mediated by oncoviruses. In this study we have investigated whether somatic alterations in CLSPN could be associated with increased breast cancer risk as well as gliomagenesis.

Material and Methods: DNA from familial (characterized for BRCA1/2 mutations) and sporadic breast cancer cases (all patients being followed at IPO Coimbra FG, EPE) and glioma patients and healthy controls was screened for mutations in CLSPN coding sequence and splice junctions using PCR and DNA sequencing.

Results: We have detected CLSPN changes in both types of cancer, namely two single nucleotide polymorphisms (Asn525Ser and IVS10+16), which co-segregated in most cases, two novel mutations (on 5’UTR-68 and codon 744), a small deletion, and one novel polymorphism (codon 6− which has meanwhile been described in another study). The 5’UTR-68 and codon 744 mutations were only found in breast cancer patients. The Gly6Asp variant was over-represented in sporadic breast cancer patients. These findings suggest the association of this variant with an increased risk for the development of breast cancer. Preliminary data have shown that co-segregation of the three polymorphisms was associated with loss of expression of Claspin in breast tumour cells, expression being retained in normal cells. These data suggest a role for Claspin as a tumour suppressor, which may be related to its role in the control of DNA replication and triggering of cell cycle checkpoint responses.

Conclusions: Our results, together with the currently available data on the role of Claspin in cell homeostasis/cancer, suggest that loss of Claspin function may be an important step in carcinogenesis.

This study was supported by FEDER and POCITI Programmes and FCT (POCTI/MCI/48912/2002; SFRH/BD/73093/2010).

[725] Genome-wide Changes of Radiation-induced Mammary Carcinoma of (Sprague-Dawley & Copenhagen) F1 Hybrid Rats Overlapping With Those of Human Breast Cancers
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Introduction: The breast tissue is one of the most susceptible organs to radiation-induced carcinogenesis. But, most studies have failed in identifying clear genetic changes in radiation-induced breast/mammary cancers. In the present study, we examined the candidate genes in the radiation-induced rat mammary cancers, which exhibited genome-wide DNA copy number changes overlapping in part with those of human breast cancers.

Materials and Methods: Forty-two 1 hybrid rats between mammary cancer susceptible Sprague-Dawley and resistant Copenhagen rats were exposed to 4 Gy gamma-rays at 7 weeks of age and underwent autopsy at the time of spontaneous death. Genome-wide DNA copy number was analyzed by array comparative genomic hybridization. Loss of heterozygosity (LOH), expression and methylation of candidate genes were also examined.

Results: Copy number loss was more frequent than copy number gain. Copy number losses were identified in small regions of chromosomes 1q52, 2q12−15, 3q31−36 and whole chromosome 5. These included the syntenic regions of human breast carcinomas. Loss of heterozygosity on chromosome 1 included genes such as Pten, Pim3,3, Fas, Map3k1, Ercc8, P21, Itiap, RbTf11, and Tp53bap1. Among these genes, expression of Fas, Ercc8 and Itiap was significantly reduced compared to those in the normal mammary gland. The loss in chromosomes 1 and 2 was either paternal or maternal with slight preference of parental origin.

Conclusions: The loss of above chromosomal regions may be causative in radiation induction of rat mammary carcinoma through dysregulation of apoptosis and interleukin 6 pathways. This rat mammary cancer model would be informative for the elucidation of the mechanism of human breast carcinogenesis.

[726] Molecular Interaction of X-rays and N-ethyl-N-nitrosourea in Thymic Lymphomagenesis Depends on the Interval of Two Treatments
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Introduction: Radiation carcinogenesis in human is considered as a result of the combined effect of two or more environmental carcinogens such as tobacco and foodstuffs. We previously showed that single exposure to X-rays or N-ethyl-N-nitrosourea (ENU) at high dose led to increased thymoma (TL) and that loss of heterozygosity (LOH) on chromosome 11, which accompanied altered expression and mutation of Ikaros, was characteristic to X-ray-induced TL (>40%), while it was rarely observed in ENU-induced ones. In addition, the combined effect of two carcinogens on the induction of TL depended upon not only the dose of carcinogen(s) but also the order of treatment. The aim of this study was to clarify the effect of the interval between X-rays and ENU on the TL induction and its underlying mechanism.

Materials and Methods: Fifty of 4-week-old female B6C3F1 mice were used in each group. They were exposed to X-rays (1.0 Gy per fraction) for four consecutive weeks, and then were treated with ENU (200 ppm) in drinking water for 4 weeks with the interval of 0, 2, 4, and 8 weeks. The mice were observed until moribundity. Lifespan, incidence of TL and LOH frequency of chromosome 11, together with that of chromosomes 12 and 19, were determined.

Results and Discussion: Lifespan of the groups with the treatment interval of 0 or 2 weeks were significantly shorter than that with the interval of 4 or 8 weeks, indicating a significant interaction of the two carcinogens with shorter interval. 2. Incidence of TL after X-irradiation alone was 13%, and that after ENU treatment was around 20% regardless of the age at exposure. The incidences of TL of the groups with the interval of 0, 2, 4, and 8 weeks were 94%, 98%, 58%, and 70%, respectively. Thus, the effect of prior X-irradiation persisted up until 8 weeks, but longer interval was much less effective than shorter one.

3. LOH frequency on chromosome 11 was low for the group with no interval (15%), suggesting less involvement of X-ray-associated LOH in TL induction. However, spilt of the two treatments longer than 2 weeks increased the LOH frequency to the level of X-irradiation alone (35−45%). LOH frequencies on chromosomes 12 and 19, however, were little affected by the presence of interval.

Conclusion: The interval period between X-rays and ENU was critical for both the frequency of TL and the mechanism of lymphomagenesis involving LOH on chromosome 11, possibly Ikaros mutation.

[727] Effects of Dietary Lipids on Growth and Puberty as a Mechanism of Their Differential Influence on Experimental Mammary Carcinogenesis
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Introduction: Breast cancer is the most frequent malignant neoplasia among women in industrialized countries. Environmental factors, especially nutritional ones, have a role in the etiology of this disease. Moreover, several evidences have demonstrated the importance of early-life events in breast cancer risk. The aim of this study was to investigate the effects of high-fat diets on
growth and maturation, mammary gland development, and its susceptibility to breast cancer.

**Material and Methods:** Female Sprague-Dawley rats were fed a low-fat, high corn oil (HCO) or high extra-virgin olive oil (EVOO) diet from weaning and gavaged with 7,12-dimethylbenz[a]anthracene. Animals were euthanized at 24, 35, 51, 100, and 246 days. We evaluated several parameters of growth and sexual maturation, as well as the clinical manifestation of mammary carcinogenesis.

**Results:** The administration of the HCO diet, but not the high EVOO diet, increased the body weight and mass of the animals. The vaginal opening was advanced in both high-fat groups, especially in HCO. This HCO group also had increased body weight around puberty, more corpora lutea at post-puberty, and tended to have higher mRNA levels of kisspeptin in the hypothalamus, a marker of sexual maturity. Both high-fat diets induced subtle modifications in the morphology of the mammary gland, with no changes on β-catenin or hormone receptors expression in the gland. The HCO diet had a clearly stimulating effect of the carcinogenesis, inducing the earliest appearance of tumors and the highest tumor incidence and yield, whereas the high EVOO diet seemed to have a weak enhancing effect, increasing tumor yield.

**Conclusion:** Our data suggest a strong influence of HCO diet in sexual maturation and mammary cancer risk, while rats fed the high EVOO diet were more similar to the controls. Moreover, the data highlight the transcension that dietetic factors may have on health and the importance of establishing healthy dietetic habits from childhood.

**[728] The Role of FOXM1 and NBS1 in DNA Double Strand Break Repair and Epirubicin Resistance**

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**Background:** Dysregulated forkhead box M1 (FOXM1) expression is associated with epirubicin resistance in breast cancer and this can occur through an enhancement of DNA damage repair. However, it still remains unclear how FOXM1 modulates DNA repair and the mechanism involved.

**Materials and Methods:** Using DNA damage and repair assays, we studied the role and regulation of FOXM1 and NBS1 in breast cancer drug resistance and sensitivity.

**Results:** Here, we demonstrated that the protein levels of FOXM1 and NBS1, which is required for activation of ATM in response to DNA double stranded break (DSB) repair, to be higher in the epirubicin-resistant MCF-7 breast carcinoma (MCF-7-EPI5) cells compared with the parental MCF-7 cells. Interestingly, the knockdown of FOXM1 by siRNA transfection significantly decreased NBS1 mRNA level in many cancer cell lines and human fibroblasts. Moreover, foxm1−/− mouse embryonic fibroblasts also reduced protein expression of NBS1 compared with wild-type mouse embryonic fibroblasts. Using DR-GFP HeLa cells, we found that depletion of FOXM1 impaired the homologous recombination-mediated DNA double stranded break repair. Furthermore, we found that foxm1−/− mouse embryonic fibroblasts transfected with wild-type FOXM1 exhibited decreased DNA breaks after epirubicin treatment, as evidenced by immunofluorescence focus staining of γH2AX, compared with foxm1−/− mouse embryonic fibroblasts transfected with control plasmid.

**Conclusions:** Taken together, our results indicate that FOXM1 mediates DNA double stranded break repair through the regulations of NBS1 expression and ATM activation.

**[729] FOXM1 Regulates BRIP1 Expression in Breast Cancer Epirubicin Treatment and Resistance**

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**Background:** Breast cancer is the most common malignancy in women, with 1 in 9 of all British and American women developing this disease in their lifetimes. Chemotherapy with anthracyclines, particularly epirubicin, plays a key role in the medical management of breast cancer. The Forkhead box M1 (FOXM1) is ubiquitously expressed in proliferating cells and its deregulation is associated with cancer progression and development of cancer drug resistance. The aim of this work is to unravel the role of FOXM1 in response to epirubicin-induced double stranded break (DSB) in breast cancer and resistant cell lines.

**Materials and Methods:** Sensitive MCF-7 and MCF-7-Epirubicin resistant (MCF-7-EPI5) cell lines were treated with epirubicin. We compared FOXM1 levels in response to epirubicin by means of Western blot and real-time quantitative PCR analysis. For the analysis of epirubicin-induced DNA damage and the influence of the drug on its repair, comet assay and immunofluorescence microscopic detection of the phosphorylated form of histone variant H2AX (γH2AX) were used. The over-expression of FOXM1 to transactivate BRIP1 promoter and to determine the FOXM1 ability to directly bind to BRIP1 promoter, luciferase and chromatin immunoprecipitation (ChIP) assays were conducted, respectively. To determine the repair pathway these genes are involved in, we performed Homologous Recombination (HR) repair assay.

**Results:** FOXM1 expression levels are maintained high in epirubicin resistant MCF-7-Epi5 cells and downregulated in sensitive MCF-7 cells following epirubicin treatment and showed a close correlation with expression of the DNA double strand break repair protein BRIP1. The epirubicin (EPI) but not the parental MCF-7 cell line shows absence of DNA damage upon epirubicin by γH2AX foci and comet assay, further, silencing of FOXM1 reverses epirubicin resistance in MCF-7-EPI5 cells and the stable FOXM1 MCF-7 cell line is able to overcome sensitivity to the same drug. Moreover, reconstituting FOXM1 in foxm1−/− mouse embryonic fibroblasts, reduces the number of foci when compared to non-transfected cells, further confirming that FOXM1 has an active role in mediating resistance to epirubicin, by enhancing repair pathways. Indeed, the knockdown of FOXM1 and BRIP1 by siRNA results in accumulation of DSBs, due to decreased repair by HR. A reporter gene assay shows that FOXM1 activates BRIP1 transcription through a forkhead-response element (FHRE) located within the proximal promoter region. The direct binding of FOXM1 to the BRIP1 promoter is confirmed in vivo by ChIP analysis.

**Conclusions:** Together, these data demonstrate that FOXM1 mediates epirubicin resistance in breast cancer in part, by transcriptionally activating DNA damage repair proteins such as BRIP1.

**[730] Analysis of EMSY in Italian Male Breast Cancer Patients**

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**Background:** Male breast cancer (MBC) is a rare disease compared to female breast cancer (FBC). MBC shares many similarities with FBC, including genetic predisposition factors such as BRCA1/2, CHEK2, PALB2, BRIP1 and RAD51C mutations. However, these alterations can explain only 10% of MBC cases, thus suggesting the contribution of additional susceptibility genes. EMSY has been recently identified as a gene involved in FBC pathogenesis because EMSY can interact with BRCA2 and in this way it is capable of silencing the activation potential of BRCA2. Moreover, breast tumors with amplified EMSY show a phenotypic profile that is similar to BRCA2-related tumors. So, because of the interaction between BRCA2 and EMSY, the latter could play a relevant role in MBC and could explain those MBC cases which pathogenesis can’t be related to BRCA1/BRCA2 mutations.

**Results:** To date, there are no information about the role of EMSY in the pathogenesis of MBC. Taking into account that EMSY has a prognostic value for FBC, studies on its role could have important implications in the elucidation of pathogenetic mechanisms of MBC and in the clinical management of MBC patients.

**Materials and Methods:** This study was performed on a series of 100 MBC cases characterized for BRCA1/BRCA2 germ-line mutations and for relevant clinicopathologic features. We have investigated the presence of germ-line mutations and amplification of EMSY by automatic sequencing and qRT-PCR respectively. Statistical analysis was performed using the Fisher exact test.

**Results:** We have found EMSY alterations in 5% of our series. Three of the 37 variants identified (M83K, M1197I and IV55–1G>A) were shown to be probably damaging by using two prediction software. We have found a general amplification percentage of 44% and we have distinguished three different amplification subgroups. A statistically significant association emerged between EMSY amplification and MIB1 (p = 0.03) expression.

**Conclusions:** Our data indicate that alterations of EMSY are involved in MBC pathogenesis at a comparable level as in FBC. New coding variants of this gene seems to be involved in MBC pathogenesis and EMSY amplification allows the identification of distinct subgroups of MBC cases. Moreover, although larger studies are needed, our results suggest that EMSY could be involved not only in MBC pathogenesis but also in tumor progression. Study supported by AIRC (IG 8713).

**[731] Gene Copy Number Alterations in Male Breast Tumors**

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**Background:** To date knowledge about specific biological and molecular characteristics of male breast cancer (MBC) is almost not existent, thus it’s difficult to identified different subclasses that have both biological and clinical relevance for breast cancer. We have analyzed a large series of MBC DNA samples to identify the occurrence of oncogenic activation in breast cancer (BC). We aimed to analyze GCN variation of genes involved in cell proliferation, hormone metabolism and cell cycle control, that
are known to play a relevant role in FBC as prognostic factors and as possible targets for therapy and that may play also a role in MBC. In particular we analyzed GCN alterations of EGFR, PIK3CA, ESR1, CCND1 and SULT1A1, in order to identify new biomarkers in MBC that may allow to better understand the pathogenesis of MBC and can lead to the identification of MBC subgroups with specific clinical-pathologic characteristics.

**Material and Methods:** GCN alterations of EGFR, PIK3CA, ESR1, CCND1 and SULT1A1 were evaluated on a series of 100 MBC tumors characterized for BRCA1/2 mutations, the major genetic risk factor, and for relevant clinical-pathologic features. The analysis was performed by TaqMan assay using Real-Time PCR.

**Results:** Overall, PIK3CA showed an amplification frequency of 8.5%, EGFR of 9%, CCND1 of 15% and SULT1A1 of 4.2%, whereas SULT1A1 and ESR1 were deleted with a frequency of 14%. Significant statistically association emerged between PIK3CA amplification and HER2 expression (p = 0.023), EGFR amplification and ER− status (p = 0.01), HER2 and MIB1 expression (p = 0.026 and 0.013) and T4 (p = 0.027). A significant statically association emerged also between CCND1 deletion and ER− status (p = 0.02), CCND1 amplification and HER2 (p = 0.017) and MIB1 expression (p = 0.03) and between SULT1A1 deletion and ER− status (p = 0.015) and G3 (p = 0.03). These data suggest that EGFR, CCND1 and SULT1A1 alterations may be linked to an aggressive phenotype in MBC.

**Conclusions:** Our results indicate that the presence of EGFR, PIK3CA, ESR1, CCND1 and SULT1A1 GCN alterations can lead to the identification of MBC subgroups with specific clinical-pathologic characteristics that can be useful in clinical management of MBC patients.

Study supported by AIRC (IG 3713).

**The p38 MAPK2-MK2 Signalling Axis is Central in the Regulation of E2F1 and FOXM1 by Epirubicin**

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**Introduction:** Elevated levels of FOXM1 are often associated with the initiation and progression of many types of cancers. In particular, FOXM1 has been reported to have a critical role in the determination of chemotherapeutic drug sensitivity. It was shown that the responsiveness of anthracycline works via the repression of FOXM1 expression which is dependent on the activity of E2F1.

**Materials and Methods:** The depletion of E2F1 expression was done using siRNA to determine the effects of FOXM1 activity upon epirubicin treatment. Further inhibition studies were done on p38 using pharmacological inhibitors, siRNAs and knockout MEFS. Phosphorylation assays were done to demonstrate the link between MK2 and E2F1.

**Results and Discussion:** We have shown that E2F1 is critical in the regulation of FOXM1 expression since its depletion by siRNA significantly affected FOXM1 induction and cell viability in response to epirubicin. Interestingly, p38-MAPK activity reflects the expression patterns of E2F1 and FOXM1 in both epirubicin sensitive and resistant MCF-7 breast cancer cells, providing a clue that p38 is involved in regulating E2F1 expression and epirubicin resistance. In agreement, results from studies using pharmacological inhibitors, siRNA knockdown and knockout MEFS revealed that p38 mediates the E2F1 induction by epirubicin and that its downstream kinase MK2 (MAPKAPK2) is the intermediary of this induction. Furthermore, in vitro phosphorylation assays showed that MK2 can directly phosphorylate E2F1 at Ser-364. Although epirubicin treatment also affects other phosphorylation events, our transfection assays also demonstrated that E2F1 phosphorylation at Ser-364 participates in its induction by epirubicin.

**Conclusions:** We have also identified Ser-364 of E2F1 as a MK2 phosphorylation acceptor-site in response to epirubicin. Collectively, these findings underscore the importance of p38-MK2 signalling axis in the regulation of E2F1 and FOXM1 expression as well as drug sensitivity in response to epirubicin. Our findings highlight the important implications for therapeutic interventions as well as predicting chemotheraphy treatment and sensitivity.

**Common Breast Cancer Susceptibility Alleles in BRCA-positive and BRCA-negative Male Breast Cancer**

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**Background:** Over the last 4 years, common low-penetration breast cancer (BC) susceptibility alleles have been reported in a total of 24 loci, identified through GWAS or candidate gene approach. Interestingly, SNPs in these loci seem also to be associated with particular clinical-pathologic features of BC, such as hormonal receptors status, and BRCA1/2 mutational status. Recently, an involvement of low-penetration alleles in male BC (MBC) susceptibility has been suggested, however, whether these loci are associated with clinical-pathologic features of MBC or BRCA1/2 mutational status is still largely unknown. Our aim was to evaluate the impact of 8 selected low-penetration alleles in MBC susceptibility, and to assess associations between BC susceptibility alleles and clinical-pathologic features of MBC, including BRCA1/2 mutational status.

**Material and Methods:** A case-control study was performed on a large MBC series collected in the first Italian multicentre study on MBC. A total of 395 MBC cases, including 46 BRCA1/2 mutation carriers, together with their clinical-pathologic characteristics, and 847 male controls, including 124 unaffected BRCA1/2 carriers.

**Results:** No significant differences were observed between the cases and controls for 7 out of 8 SNPs. Only rs2981582 (per allele OR, 1.67; 95% CI, 1.41–1.98) showed a statistically significant association (p < 0.05) emerged between BRCA2-negative MBCs, and between 841 male controls and 847 unaffected BRCA1/2-positive MBCs. ESR1 was indeed associated with PR−, HER2−, higher tumor grade and absence of BC family history.

**Conclusions:** Overall, based on a large multicentre series, our data support the hypothesis that common low-penetrance BC susceptibility alleles play a role in MBC susceptibility. Moreover, our results suggest that specific loci may be associated with distinct MBC subtypes and may act as genetic modifiers of BRCA2 in men.

**Hepatitis C Viral Proteins Modulate Apoptosis and Inflammation Through Bid Protein**

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**Introduction:** Hepatocellular carcinoma (HCC) is the 5th most common cancer and the 3rd cause of cancer related mortality worldwide. Viral infection by hepatitis C (HCV) or B (HBV) viruses is by far the most frequent etiology of HCC. Because of dramatic lack of efficient therapies for HCC, prevention remains the utmost priority for hepatitis C patients. Among the multiple viral strategies to avoid elimination by the host immune response, HCV modulates the apoptotic response of hepatocytes by downregulating Bid, a BH3-only protein of the Bcl-2 family, rendering the cell resistant to the extrinsic apoptotic signalling and thus to elimination by cytotoxic T lymphocytes.

It has recently been shown that Bid also participates in the control of innate immune response. It interacts with NOD intracellular receptors, which are involved in both anti-microbial and anti-viral response. In view of the importance of the inflammatory context for tumorigenesis, the direct effect of HCV proteins on these innate immunity receptors is likely to be highly relevant to the initiation and progression of HCC.

**Material and Methods:** We have used an in vivo model of HCV transient release (HCV-N305) and cellular models of primary human hepatocytes infected with HCV as well as HepaRG and Huh-7 cell lines. Expression of Bid has been assessed by western blots and RT-qPCR and its consequences by apoptosis assays. Stimulation of NOD and RIG-I pathways has been achieved by treatment with appropriate ligands and assessed by quantification of inflammatory cytokines.

**Results and Discussion:** HCV protein NS5A activates cellular cytokines that degrade Bid. Interestingly, pharmacological inhibition of calcines restores...
Multidrug Resistance Transporter Pgp as a Pathogenetic Factor of Non-small Cell Lung Cancer

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Conclusion: Moreover, we were able to demonstrate for the first time that NOD1 pathway constrains tumor progression in colorectal cancer via induction of apoptosis. This study to the role of NS5A and Bid in inflammatory signalling, we have shown an increase in NOD1 mRNA expression in transgenic mice. This result was confirmed in primary human hepatocytes infected with HCV as well as in a cell line harboring an HCV replicon. Moreover, we were able to demonstrate for the first time that NOD1 pathway is active in human hepatocytes.

Material and Methods: To specifically adress the role of DCC-induced apoptosis in tumorigenesis and to bypass perinatal death of DCC knockout mice, we created a mouse model in which DCC’s pro-apoptotic activity is genetically silenced by introduction of a point-mutation in its intracellular domain.

Results: While the loss of DCC-induced apoptosis in this mouse model is not associated with a major disorganisation of the intestines, it leads to a decrease of basal intestinal apoptosis and to occurrence of spontaneous intestinal neoplasia at a relatively low frequency. Loss of DCC-induced apoptosis is also associated with an increase in the number and the aggressiveness of intestinal tumors in a predisposing APC mutant context, resulting in the development of highly invasive adenocarcinomas.

Conclusions: These results demonstrate that DCC functions as a tumor suppressor, via its ability to trigger tumor cell apoptosis.

Evaluation of Anti-angiogenic Therapy on the Effects of Angiogenic and Apoptotic Pathways in Breast Cancer Cell Line

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Breast cancer is one of the most common cancer types in the world. In various types of tumors, cell death plays an important role in the process of cancer growth and metastasis. Breast cancer is one of the most common cancer types in the world. In various types of tumors, cell death plays an important role in the process of cancer growth and metastasis. Cell death in tumours is commonly attributed to the induction of apoptosis. In recent studies, it is reported that one of the anti-vomiting drugs, thalidomide, inhibits angiogenic mechanisms. At the same time it is reported that propranolol treatment, the beta blocker drug, shrinks in hemangiomas in infantile hemangio ma cases. In this study our aim was to investigate the effects of anti-angiotherapeutic (Pacitaxel [PX]) and anti-angiogenic treatment (Thalidomide [TD] and Propranolol [PR]) on the distribution of the Vascular Endothelial Growth Factor (VEGF) and Caspase 3 in the invasive human breast cancer cell line (MDA-MB-231). After 72h treatment with drug, the cells were stained for VEGF and Caspase 3. The cells were treated with PX (10 μM), PR (70 μM) and TD (75 μM). PX+PR and PX+TD groups. The drug effects at the 48th hour were evaluated. After fixing the cells with paraformaldehyde, the avidin-biotin peroxidase method was employed by using the anti-VEGF and anti-Caspase 3 primary antibodies. Immunohistochemical distribution intensities of primary antibodies were scored as minimal (−), mild (+), moderate (+++) and strong (++++) and analyzed by using the statistic ANOVA test. It was observed that, the immunoreactivity of VEGF was observed as strong, moderate, mild and minimal in the control, PX, PR and TD groups, respectively. Immunoreactivity of Caspase 3 was observed as strong in the PX group, mild in the PR and TD groups and minimal in the control group. In the PX+PR and PX+TD groups, the immunoreactivity of VEGF was decreased compared to the control and PX groups. In these groups, the immunoreactivity of Caspase 3 was similar compared to the PX group and increased compared to the control, PR and TD groups. Anti-angiogenic drugs such as Propranolol and Thalidomide in addition to the anti-chemotherapeutic therapy might less effective on the apoptotic pathways, but might inhibit the tumor invasion and metastasis by affected angiogenic mechanisms. Thus progressive inhibition of angiogenesis may contribute to tumor initiation, growth and metastasis in the breast cancer.
**Cancer Therapy Models – Development of a Novel Clinically Relevant Orthotopic Xenograft Rat Model of Metastatic Colorectal Adenocarcinoma**


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**Introduction:** The failure rate of phase-3 trials are high in oncology than in other therapeutic areas, reinforcing the desire to develop more useful animal models that closely replicate the histology, physiological effects, biochemical pathways and the behavior of the patient's human tumors.

The orthotopic model (ORT) of human tumors in immunosuppressed mice models that closely replicate the histology, physiological effects, biochemical microenvironment, slow progression of symptoms, greater longevity and less metastatic potential. The orthotopic model of colorectal adenocarcinoma (CRC) is in the cecum. As this is an unusual site in humans it becomes necessary to establish other ORT model of CRC, in the left colon, through microsurgery with micro-injection of human CRC cell line WiDr in the mucous fistula, and compare both models. The progression assessment was done resorting to nuclear medicine techniques, using 111Tc-MIBI and 125I-FDG.

**Methods:** 31 RNU rats underwent two surgical procedures (cecostomy and WiDr cells injection) and injected with WiDr cells (10^14–10^15 cells/animal), after return to normal bowel function. Evaluation with 111Tc-MIBI and 125I-FDG were performed after intravenous injection and acquired using a gamma-camera and a prototype ClearPEM, respectively.

**Results:** For equal amount of cells inoculated in both models, coloresterom- induced model showed higher longevity and better life quality, expressing slow progression of symptoms, contrasting to animals with cecostomies.

**Discussion/Conclusion:** These preliminary data suggests that the coloresterom model seems to be the best model of CRC that in characterizing tumor microenvironment, slow progression of symptoms, greater longevity and less metastatic potential. Although good tumor tracers, the different characteristics of the radiopharmaceuticals used have clear advantages and disadvantages that can influence the interpretation.

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**Growth Factor Midkine is Associated With Castration-resistant Prostate Cancer and Neuroendocrine Differentiation**

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**Background:** Castration-resistant prostate cancer (CRPC) is an incurable disease and the molecular changes responsible for transition from hormone-naïve (HN) PC to the aggressive CRPC state remains to be fully understood. CRPC transition is closely associated with both androgen-deprivation therapy (ADT) and neuroendocrine differentiation (NED), and more knowledge concerning neuroendocrine (NE)-transformed PC cells, the NED process and CRPC progression is needed.

**Materials and Methods:** Immunohistochemical analysis of MDK and tUB3 in HN and NE- Markers positive tumor samples from a total of 62 patients were analyzed for MDK and tUB3 expression. Triple immunofluorescent imaging was performed on a selection of specimens.

**Results:** MDK, tUB3 and CA9 were upregulated in CRPC compared to HN tumors. MDK was highly associated with both CA9 and tUB3 staining, while CA9 and tUB3 staining only displayed a partial expression overlap, an overlap almost exclusively displaying also MDK expression. MDK-positive NE-like looking cells were identified in malignant prostate tissue and found to co-express CA9 alone or, more commonly, CA9 together with tUB3.

**Conclusions:** Upregulation of MDK during PC progression is associated with CRPC and NED (shown by its relation to CA9). The upregulation of tUB3 in CRPC is associated with MDK and to an extensive population of CA9+MDK+ expressing NE-like tumor cells. The results suggest that MDK is involved in the NED process, possibly also representing an over-bridging marker between different subpopulations of NE-like tumor cells, something that needs to be evaluated experimentally as does the applicability of MDK as a future target for CRPC.

**Ongoing studies aim to evaluate MDK and tUB3 expressions during steroid deprivation-induced apoptosis. Preliminary results link MDK expression to NED (NE-differentiated PC cells) and a castration resistant expression profile also in vitro. The role of MDK during this upregulation is under current investigation.**

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**Liver Metastasis, Evaluated From the HGF/c-Met Pathway**

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Colorectal cancer (CRC) is the third most frequent malignancy, but the second cause of death for tumor in the western population.

Chemotherapy relies essentially on the induction of apoptosis by DNA damage, but impairment of apoptotic and/or anti-apoptotic genes causes the acquisition of drug resistance. Thus, a few selective compounds are currently used for CRC treatment to circumvent drug resistance and reduce side effects, while others are now under clinical trials.

In recent molecular studies several that showed cross-talking pathways are deregulated in CRC, including cell growth, differentiation and death. Microarray analysis and different mutants and inhibitors in combination with molecularly targeted drugs are now under clinical trials for inflammatory diseases and cancer.

In this work we tested p38 inhibitors in combination with molecularly-targeted drugs and chemotherapeutic agents in CRC xenografted nude mice and the AOM/DSS colitis-associated carcinoma preclinical model. To this aim, animals were treated with SB202190 alone or in combination with the orally administrable MEK1 inhibitor PD0325901, the BRAF inhibitor Sorafenib or the chemotherapeutic agent Cisplatin (CDDP). The combined use of p38 inhibitor with the indicated compounds significantly reduced tumor growth by inducing apoptosis throughout the treatment compared with each single treatment. These data validate in vivo the combined inhibition of p38a and MEK1 as a promising approach to reduce CRC growth by inducing apoptosis. We believe that p38 inhibitors will prove a valid approach for future combined therapy with chemotherapeutic agents and molecularly targeted drugs.

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**Clinical Significance of Aggressive Hepatocyte for Colorectal Liver Metastasis, Evaluated From the HGF/c-Met Pathway**

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**Background:** Liver metastasis is one of the most critical factors in deciding the prognosis of patients with colorectal cancer (CRC). Hepatocyte is the most common type of treatment for liver metastasis of CRC. The high amount of hepatocyte growth factor (HGF) is produced to promote liver regeneration by hepatocyte. Theoretically, HGF produced after hepatocyte stimulation promotes the progression of CRC cells with c-Met in residual liver. This study was aimed to evaluate the value of hepatocyte towards liver metastasis of CRC in relation to the HGF/c-Met pathway.

**Material and Methods:** Ninety-four patients with CRC (including 24 liver metastasis cases) were operated at Gifu University Hospital (2002–2004). For
these cases, the expression of c-Met in the primary and liver metastatic sites was evaluated by immunohistochemistry and Western blot. Experiments were also conducted on C126 murine CRC cell line and a mouse liver metastasis model.

**Results:**
In clinical study, the c-Met expression in liver metastatic sites was lower than that of primary sites in 87% of 24 cases. In basic study, the expression of c-Met protein in the liver tumor was significantly lower than in culture cells according to Western blot (p = 0.033). The growth of residual liver tumors was not significantly different between 30% heptectomy group and no operation group. The over-expression of c-Met was closely associated with CRC liver metastases. On the other hand, in liver metastatic lesions, the c-Met expression was reduced in comparison to primary lesions.

**Conclusion:**
Even if serum HGF levels increased due to liver resection during the regeneration period, residual liver metastases of CRC was not promoted in its progression. Aggressive hepatocellular carcinoma would still be acceptable and favorable as a curative therapy.

**[747] Dynamin 2 Overexpression Correlates with Prostate Cancer Mortality in Patients and Regulates Focal Adhesion Dynamics in Invasive Prostate Cancer Models**

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**Background:**
Cell signaling that control prostate cancer (PCA) progression to hormone refractory PCA and metastasis remain partially understood. Here we investigated the role of Dynamin 2 (Dyn2) in PCA development and progression. Dyn2 is a member of the large GTPase family essential for vesicle formation in receptor-mediated endocytosis, synaptic vesicle recycling, caveolar internalization, and possibly vesicle trafficking in and out of the Golgi. In particular, Dyn2 plays an important role in clathrin-mediated endocytosis/recycling of several oncogenic receptors but it can also impact on the regulation of cell cycle and angiogenesis.

**Study design:** We evaluated Dyn2 protein expression in two large cohorts of localized and castration-resistant PCA. This was complemented by in vitro studies using androgen-responsive and androgen non-responsive PCA cells where Dyn2 gene was silenced by shRNA to evaluate the effects of Dyn2 inhibition on cell migration, cell invasion, and FA signalling and turnover in live cells and real-time. The impact of Dyn2 inhibition on PCA progression to metastasis was investigated in animal models.

**Outcome:**
Immunohistochemistry analysis of 1260 cores from PCA tissues isolated from 326 patients revealed that Dyn2 protein expression is significantly increased in neoplastic epithelia and across advanced stages of PCA compared to benign tissue. Overall mean expression values in high-grade intraepithelial neoplasia (HPGIN) (n = 48, 2.21 ± 0.54), localized PCA (n = 821, 2.13 ± 0.79) and castration resistant PCA (CRPC) (n = 199, 2.37 ± 0.78) were significantly higher compared to benign prostate tissue (n = 192, 0.8 ± 0.74) (p < 0.0001). Higher Dyn2 expression in CRPC was associated with PCA specific mortality (p = 0.048). In preclinical PCA models, Dyn2 gene silencing in the androgen-responsive cell line LNCaP and androgen non-responsive cell lines C4-2 and PC3 significantly reduced cell migration and invasion in vitro compared to control cells; these phenotypes were associated with enhanced expression of focal adhesion proteins including vinculin, paxillin, and focal adhesion kinase at plasma cell protrusions. Live cell imaging revealed a drastic inhibition of focal adhesion turnover at cell protrusions of migratory cells. Dyn2-silencing in PCA cells also resulted in a significant decrease in tumor size (p < 0.01) and lymph node metastases (p < 0.005) when cells were implanted into the prostate of immunocompromised mice.

**Conclusion:** This data support a role of Dyn2 overexpression in PCA carcinogenesis and poor prognosis. These results also highlight the potential of Dyn2 to serve as a therapeutic target for advanced PCA.

**[748] Relationship Between Histology and Telomere Status in Non Small Cell Lung Cancers – New Approach for Molecular-targeted Therapies**

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**Introduction:** Senescence and cell death pathways develop an important role limiting tumor growth in response to short terminers. The failure of factors related to these pathways could explain the poor clinical outcome conferred by telomere shortening in Non Small Cell Lung Cancers (NSCLCs) [1]. With the aim of determining the molecular base which would explain this find, we identified a small genetic sign (TP53, DAPK1, GADD45A and SHC1), related to these pathways, which was downregulated in the group of patients whose tumors showed a clinical telomere shortening. Taking into account the advent of successful new molecular-targeted therapies for lung cancer, many of which are associated with specific histologic cell types and subtypes, our first aim consists of defining the role of telomere status as prognostic factor with relation to histologic types, and the significance of the small genetic sign, previously identified, in this context. **Material and Methods:** We analyzed 8 NSCLCs and their corresponding control tissues, obtained from patients who had undergone potentially curative surgery. Telomere function was evaluated by determining telomerase activity and telomere length. The Telomere Restriction Fragment (TRF) length ratio was determined as the ratio of the length of tumor tissue TRF and their paired normal tissue TRF (T/N ratio). All cases in which the T/N ratio was < 1 were placed in the group of telomere shortening. When the T/N ratio was ≥ 1, tumors were placed in the group of telomere maintenance. Gene expression levels were determined by real-time quantitative PCR (RT-qPCR) for DAPK1, GADD45A, SHC1 and TP53.

**Results:**
The mean telomere length (mean ± typical error) was 4.51 ± 0.33 Kbp in NSCLCs and 5.44 ± 0.32 Kbp for nontumor samples, and the mean telomere length ratio T/N was 0.83 ± 0.029. Telomere shortening was showed by 73.2% of the tumors and 26.8% maintained telomere maintenance. We observed significant association between telomere status and histology of tumors (p-value = 0.048). Transcriptional expression for DAPK1, GADD45A, SHC1 and TP53 was higher in squamous cell carcinomas with telomere maintenance with regard to those showing telomere shortening (significant results for DAPK1 and GADD45A).

**Conclusion:** Our results offer the possibility to sub-classify squamous cell carcinomas according to telomere status, which defines different molecular profiles useful for personalized treatment.

**Reference(s)**

**[749] Myc – a Non Redundant Function in Cancer**

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**Background:** In theory, the ideal cancer drug should target a non-redundant function required to drive tumor cell growth and survival yet be dispensable for all normal cells. Hitherto, the usual strategy for identifying cancer drug targets has been to identify the recurrent mutations in each cancer type on the basis that such recurring mutations play an exclusive and irreplaceable role in tumour maintenance. However, whilst the same general pathways are activated in most cancer types, such activation is typically mediated by mutations in highly functionally degenerate and homeostatically robust signaling networks: hence, the therapeutic impact of their inhibition is rapidly degraded by compensatory adaptation or evolution. The Myc transcription factors are activated, usually indirectly, in most, perhaps all cancers. We previously showed in a preclinical mouse model of lung cancer that systemic Myc inhibition has a dramatic therapeutic impact, triggering ostensibly complete regression of tumors with only mild and fully reversible side-effects on normal tissues.

**Materials and Methods:** Making use of the same preclinical mouse model of lung cancer and Myc inhibitory transgene, we assessed tumor recurrence upon disruption of signaling. These results for

**Conclusion:**
We now provide evidence that Myc is indeed a unique and non-degenerate function in cancer that cannot be replaced by other pathways, even in the most aggressive p53 null tumors.

**Conclusions:** Myc could be targeted safely and successfully without eliciting resistance to therapy.
therapy. Aim of this study was to elucidate the role of IL-8 in ER+ breast cancer progression in untreated and patients treated with tamoxifen.

Material and Methods: This study involved 150 postmenopausal breast cancer patients with known clinicopathological characteristics and detectable levels of ER. 91 patients didn’t receive any kind of treatment (median follow up time was 45 months) and 59 were treated with tamoxifen (median follow up time was 147 months). IL-8 levels were determined by ELISA in primary tumor tissue lysates according to manufacturer instruction (RayBio Human IL-8 ELISA kit). Results: There was no correlation between IL-8 expression and available clinicopathological parameters, except for strong negative correlation between IL-8 and ER (p = 0.0001) for the whole cohort of patients. Unexpected, untreated patients had a better disease free survival than tamoxifen treated (p = 0.008) although untreated subgroups had higher IL-8 levels than tamoxifen treated (p > 0.001). Stratification of untreated and treated patients, revealed that there was significant difference in disease free survival between subgroups (according to corresponding median IL-8 levels) in untreated group (p = 0.003), but there was no significant difference between different IL-8 subgroups in tamoxifen group (p = 0.5). Untreated patients with higher levels of IL-8 had the worse prognosis. Median IL-8 level in untreated group was 71.7 pg/mg and in tamoxifen treated was 9.1 pg/mg. Considering the negative correlation between ER and IL-8 and the effect of tamoxifen that blocks the effects of ER, there is a possibility that during treatment period, consequently, tamoxifen could increase IL-8 levels and therefore induce a worse outcome of tamoxifen treated patients.

Conclusion: Our results deserve further investigations, because this finding candidate IL-8 for marker of tamoxifen resistance. However, beside its undefined predictive value, it seems that IL-8 is a strong and independent prognostic parameter for untreated breast cancer patients.

751 MYC Inhibition as a Therapeutic Strategy in Glioma

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Background: Gliomas are the most common primary tumors affecting the adult human Central Nervous System (CNS). The most lethal is grade IV glioblastoma (GBM), which gives a median survival of only 15 months, but also less severe grades respond poorly to standard therapy. Myc is a BHHLZIP transcription factor, causally implicated in most human tumors. In the past, we employed a dominant negative Myc transactivation activity, termed Ommomyc, and showed that repression of Myc was a strong feature in glioblastoma, both in K-Ras12;Ptf1a driven lung tumors and in T antigen driven pancreatic β-cell insulomas.

Materials and Methods: We made use a mouse model of Ha-Ras driven glioblastoma and of our Ommomyc switchable model in order to demonstrate Myc inhibition as a therapeutic strategy in glioma. We also tested neuroprogenitors cells derived from the same animal models, as putative cells of origin of glioma. Finally we tested Myc inhibition in human glioblastoma cell lines and patient derived samples.

Results: Myc inhibition has a dramatic therapeutic impact on Ha-Ras driven gliomas in both mouse models and human cells, being able to arrest tumor progression and causing regression, remission and cure of murine and human tumors.

Conclusions: Myc inhibition can be a valuable therapeutic approach in the treatment of gliomas.

752 Phytotherapy with Angiosperms From Arid Zones of Northern Mexico in Patients With Malignant and Benign Tumors

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Background: At the arid and semiarid regions from northern Mexico, diverse angiosperms are profusely applied in ethnomedicine for the treatment of not specified neoplasia. A phytotherapeutic approach as sole therapy, was implemented in a pilot group of patients with malignant and benign tumors using mixtures of aqueous extracts of various angiosperms with antineoplastic basic activity. The basis of this approach is the translational research, typically implementing mixtures of aqueous extracts of various angiosperms with antineoplastic activity. The basis of this approach is the translational research, typically using mixtures of aqueous extracts of various angiosperms with antineoplastic activity. In this pilot group of patients with malignant and benign tumors, phytotherapy was prescribed as only casual treatment. No surgical drainage was made of the effusion. On June 24, 2010, the pleural effusion vanished. Patient was discharged. February 12, 2012, remains asymptomatic.

Conclusions: The mixture of aqueous extracts of angiosperms from the deserts of northern Mexico, prescribed as a single oral treatment, has antineoplastic activity in both benign and malignant tumors. This preliminary phytotherapeutic approach has demonstrated no adverse reactions or clinical and laboratory events, improving quality of life and survival.

753 Cytokeratin-20 as a Marker for Detection of Circulating Tumor Cells in Preoperative and Postoperative Blood Samples from Colorectal Cancer Patients

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Background: In this study cytokeratin-20 (CK-20) was evaluated as a marker for detection of circulating colorectal cancer cells by real-time RT-PCR and influence of surgical tumor resection on the presence of circulating colorectal cancer cells was analyzed.

Material and Methods: Blood samples were taken from 95 colorectal cancer patients before and after tumor resection. Blood samples from 23 healthy volunteers were analyzed as controls. RNA was isolated from mononuclear cell fraction of the blood and assayed by real-time RT-PCR for the expression of CK-20.

Results: One out of 23 healthy volunteers was positive for CK20. Out of 95 colorectal cancer patients, 25 were positive for CK20 before surgery and 23 were positive for CK20 after surgery. Nine patients that were positive before surgery became negative after surgery, while 15 patients that were negative before surgery became positive after surgery. A statistically significant trend of increase in the proportion of CK-20 positive blood samples with increasing stage of the disease was observed for preoperative, but not for postoperative blood samples.

Conclusions: This study confirmed that CK-20 is a specific marker for detection of circulating colorectal cancer cells and association with clinical stage suggests that it might be used in prognosis. We have also shown that surgical tumor resection can both reduce and induce the presence of circulating colorectal cancer cells.

754 Vascular Endothelial Growth Factors Protein Overexpression in the Prediction of Early Relapse of Colorectal Cancer Patients

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Background: Angiogenesis plays an important role in progression of colorectal cancer (CRC), and evidences from preclinical and clinical studies indicate that vascular endothelial growth factor (VEGF) is the predominant angiogenic factor. The purpose of this study was to identify this novel predictors of early relapse in UICC stage I-II CRC, and further to determine its correlation to disease outcomes.

Materials and Methods: Clinicopathological features and VEGF expression by immunohistochemical staining were retrospectively analyzed in 100 UICC stage I-II CRC patients undergoing curative resection to determine the predictors of postoperative early relapse. Results: Of 100 CRC patients, forty patients and sixty patients were categorized to early relapse and non-early relapse, respectively. Multivariate logistic regression analysis showed that vascular invasion (P = 0.048), perineural invasion (P = 0.042), VEGF overexpression (P = 0.023), and high postoperative carcinoembryonic antigen (CEA) level (P = 0.004) were demonstrated to be independent predictors of early relapse. A combination of vascular invasion, perineural invasion, VEGF overexpression and postoperative CEA level as predictors of early relapse showed that the more predictors that are involved, the higher chance that postoperative early relapse would occur. Moreover, VEGF overexpression could not only predict the possibility of postoperative early relapse but also affect the disease-free survival (P < 0.001) and overall survival (P = 0.002) rates.
Conclusions: This study suggests that VEGF protein overexpression is a crucial predictor of postoperative early relapse in UICC stage III-C CRC patients and that this could help to define patients who would benefit from enhanced follow-up and therapeutic programs.

Poster Sessions

From Genome-wide Association Studies to Translational Genomic Discord of Bladder Cancer

L. Prokunina-Ólsson1, Y.P. Fu1, W. Tang1, J.D. Figueroa1, I. KohaaS, J. Ea2, D.T. Silverman1, X. Wu3, F. Re4, N. Rothman5, 1. National Cancer Institute, Laboratory of Translational Genomics, Bethesda MD, USA, 2. National Cancer Institute, Division of Cancer Epidemiology, Bethesda MD, USA, 3. National Institute of Mental Health, Bethesda MD, USA.

Conclusions: The dataset initially consisted of 174 paraffin-embedded neuroblastoma samples. Further research is required to determine whether neuroblastoma samples of different ages can be validated using this technique.

Loss of IGF-binding Protein 3 of EGFR Mutant Lung Cancer Cells in Acquired Resistance to EGFR-tyrosine Kinase Inhibitor

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Introduction: Acquisition resistance inhibitors (EGFR-TKis) and EGFR resistance effectors are thought to be candidate mechanisms of EGFR-TKI resistance. As such, we performed a study to detect expression of IGFBP-3 in cell lines with ESRT resistance. We found that the GWAS signal within the 18q12.3 region of paraffin-embedded neuroblastoma samples is suggestive as one of possible resistant mechanisms in the 1431 and HN115 cell line.

Material and Method: Here, we investigated whether the loss of IGFBP-3 affects the acquired resistance to EGFR-TKis and if so, it could be useful as a marker for development of resistance. In addition, the effect of induced IGFBP-3 on overcoming resistance was evaluated.

Results: Results sublines of HCC827 to gefitinib (HCC827/GF3), erlotinib (HCC827/ER3) and CL387.785 (HCC827/CLR) were established by chronic exposure to drugs. Loss of IGFBP3 expression was commonly found by Western blots without change of transcriptional activity in all of them regardless of resistant mechanism. Supporting this, the amount of secreted IGFBP3 was significantly reduced in culture media by ELISA. Desirably, loss of IGFBP-3, there was no change of IGF-3 signals. Forced expression of IGFBP-3 by transfection of adenosine-IGF-binding protein 3 and addition of recombinant IGFBP-3 showed only modest effects on the enhancement of growth-inhibitory and apoptotic effect of EGFR-TKis. Serum IGFBP-3 levels before EGFR-TKI and after resistance in 20 patients were analyzed by ELISA. There was no significant change of IGFBP-3 level (1815.3±94.6 ng/ml before EGFR-TKI vs 1779.8±87.8 ng/ml after resistance).

Conclusion: In summary, although loss of IGFBP-3 is a common accompanying phenomenon occurred during acquisition of resistance to EGFR-TKI regardless of mechanisms, its effect on resistance was only modest suggesting that it would not act as a primary major role on resistance. In addition, may it not be a useful serum marker indicating resistance.

Functional Characterization of NK- and T-cells in IFN-a Monotherapy Treated Chronic Myeloid Leukemia (CML) Patients

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Introduction: CML is caused by a constantly active tyrosine kinase BCR-ABL Tyrosine kinase inhibitor (TKI) therapy has improved the prognosis of CML significantly. Before the TKI era, CML patients were treated with interferon-α (IFN-α), but only a small proportion of patients responded well. However, about half of the responding patients eventually developed disease relapse. The mechanism of cure is unknown. Therefore, we aimed to study the function of NK- and T-cells in IFN-α monotherapy treated CML patients to understand the putative anti-leukemic effects.

Material and Methods: Due to the rarity of IFN-α monotherapy treatment nowadays, the study included only 13 CML patients who were in complete remission for at least 6 months, 5 patients were currently using IFN-α (IFN-ON) and 8 had stopped treatment successfully (IFN-OFF). Samples from 10 healthy volunteers were used as controls. The cytotoxicity of NK-cells was studied by measuring direct killing and degranulation (CD107a) assay using K562 as targets. The function of T-cells was studied by antibody (OKT3) stimulation and measuring TNF-α and IFN-γ production by flow cytometry. In addition, NK-cells were phenotyped by a multi-color flow cytometry.

Results and Discussion: In CML patients, the cytotoxic potential of NK-cells was similar as in healthy controls. NK-cells from IFN-ON patients expressed more C6DE2 (47.4%) than IFN-OFF patients (24.3%) or healthy controls (29.3%; p = 0.14), but less CD57 (33.7%, 70.6%, 61.2%; p = 0.15). IFN-ON patients also had a higher proportion of interferon regulatory factor 3 (IRF3) positive cells than IFN-OFF patients in a higher number of NK-cells in IFN-ON patients than IFN-OFF patients and healthy controls.

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Xenospheres – a Preclinical Model of Tumor-initiating Cells to Study the Response to Targeted Therapies in Metastatic Prostate Cancer

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The use of targeted therapies for cancer treatment has significantly grown in the field of cancer research. The use of targeted therapies for cancer treatment has significantly grown in the field of cancer research. The use of targeted therapies for cancer treatment has significantly grown in the field of cancer research. The use of targeted therapies for cancer treatment has significantly grown in the field of cancer research.

Prognostic and Predictive Value of TP53 Mutations in Prostate Cancer

S. Weng1, D. Tracey-White1, O. Argyros1, R. Harbottle1. 1Imperial College London, National Heart and Lung Institute, London, United Kingdom

Background: The development of genetically marked tumour xenografts is a field of active research enabling an easier and more reliable testing of cancer therapies and the analysis of the mechanisms of tumorigenesis. Genetically marked tumour models have a number of advantages over conventional tumour models, including the easy longitudinal monitoring of therapies and the reduced number of animals needed for trials.

Methods: Several different methods have been used in previous studies to mark tumours genetically, however all have limitations, such as genotoxicity and other artefacts related to the use of integrating viral vectors. Recently we have generated an episomally maintained plasmid DNA (pDNA) expression system based on Scaffold/Matrix Attachment Region (S/MAR), which overcomes these problems and provides permanent genetic modification and persistent transgene expression in tumour cells without these limitations.

Results and Discussion: Here we describe the use of this pDNA vector to create human hepatoma (HuH7), pancreatic carcinoma (MIA PaCa-2) and Birt-Hogg-Dubé kidney folliculin (FLCN) knockout (UOK257) cell lines stably and genetically marked with the luminescent reporter gene luciferase. These modified cell lines were xenografted into NOD-SCID mice and monitored longitudinally over time using a Xenogen imager. Each cell line exhibited the permanent episomal maintenance of the vector and sustained long-term luciferase transgene expression. Importantly, each formed tumours showing the respective pathological characteristics of hepatocellular carcinoma, pancreatic carcinoma and renal cancer.

Conclusion: This is the first demonstration that a non-viral, replicating, non-integrating pDNA vector can confer sustained transgene expression in various murine tumour models. We believe that this system provides great utility and advantages over currently used technologies and will be a useful resource to the field of cancer research.

Phosphorylation of the Androgen Receptor by Cdk1 Predicts Outcome in Prostate Cancer Patients with PSA < 20 ng/ml

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Material and Methods: Scansite 2.0 was utilized to predict Cdk1 and MAPK consensus sites on AR including Ser-81, 94, 308, 515 and 650. To assess expression of Cdk1, pCdk1, pMAPK, AR and AR phosphorylation at predicted sites in clinical samples, immunohistochemistry was performed on 90 patients with hormone naïve prostate adenocarcinoma recruited at a referral centre between 1992–2002. Biochemical relapse, survival from biochemical relapse and disease-specific survival according to high/low protein expression were analysed using Kaplan–Meier methods. Significant findings were included in a co-regression model. Pearson’s rank correlation coefficients (c.c) were calculated to assess association between protein expression.

Results: High cytoplasmic pAR515 correlated with decreased time to biochemical relapse. High cytoplasmic pAR515, Cdk1 and nuclear pCdk1 correlated with decreased disease-specific survival. In a co- regression model, high cytoplasmic pAR515 and serum PSA at diagnosis were independently associated with biochemical relapse. In patients with serum PSA < 20 ng/ml at diagnosis and high pAR515 expression the proportion that experienced biochemical relapse at 5 years was 77.5% vs 29.1% (p = 0.019). This translated to shorter 10 year disease-specific survival 38.1% vs 100% (p = 4 x 10^-6). Cdk1 and/or pCdk1, but not pMAPK, were correlated with expression of all predicted AR serine phosphorylation sites. Nuclear pCdk1 was associated with nuclear pAR515 expression (c.c 0.558, p = 9.5 x 10^-4).

Conclusions: Phosphorylation of AR at Ser-81, 94, 308, 515 and 650 is associated with nuclear pCdk1 and pCdk1, but not MAPK. Expression of cytoplasmic pAR515 may act as an independent prognostic marker and inform treatment decision-making in prostate cancer patients with serum PSA < 20 ng/ml at diagnosis.

Genetic Modification of Cancer Cells Using Non-viral Vectors - Harbouring a Scaffold/Matrix Attachment Region

S. Wang1, D. Tracey-White1, O. Argyros1, R. Harbottle1. 1Imperial College London, National Heart and Lung Institute, London, United Kingdom

Background: The development of genetically marked tumour xenografts is a field of active research enabling an easier and more reliable testing of cancer therapies and the analysis of the mechanisms of tumorigenesis. Genetically marked tumour models have a number of advantages over conventional tumour models, including the easy longitudinal monitoring of therapies and the reduced number of animals needed for trials.

Methods: Several different methods have been used in previous studies to mark tumours genetically, however all have limitations, such as genotoxicity and other artefacts related to the use of integrating viral vectors. Recently we have generated an episomally maintained plasmid DNA (pDNA) expression system based on Scaffold/Matrix Attachment Region (S/MAR), which overcomes these problems and provides permanent genetic modification and persistent transgene expression in tumour cells without these limitations.

Results and Discussion: Here we describe the use of this pDNA vector to create human hepatoma (HuH7), pancreatic carcinoma (MIA PaCa-2) and Birt-Hogg-Dubé kidney folliculin (FLCN) knockout (UOK257) cell lines stably and genetically marked with the luminescent reporter gene luciferase. These modified cell lines were xenografted into NOD-SCID mice and monitored longitudinally over time using a Xenogen imager. Each cell line exhibited the permanent episomal maintenance of the vector and sustained long-term luciferase transgene expression. Importantly, each formed tumours showing the respective pathological characteristics of hepatocellular carcinoma, pancreatic carcinoma and renal cancer.

Conclusion: This is the first demonstration that a non-viral, replicating, non-integrating pDNA vector can confer sustained transgene expression in various murine tumour models. We believe that this system provides great utility and advantages over currently used technologies and will be a useful resource to the field of cancer research.
doxorubicin-based chemotherapy with or without docetaxel [ClinicalTrials.gov identifier: NCT00174655].

Material and Methods: The prognostic and predictive values of TP53 were analyzed in tumor samples by gene sequencing within exons 5–8. Patients were classified according to p53 protein status predicted from TP53 gene sequence, as wild type (no TP53 variation or TP53 variations which are predicted not to modify p53 protein sequence) or mutant (p53 non-synonymous mutations). Mutations were subclassified into missense or truncating mutations. Survival analyses were performed using Kaplan–Meier method and log-rank test. Cox-regression analysis was used to identify independent predictors of outcome.

Results: TP53 gene status was determined for 18% (520/2887) of the women enrolled in BIG 02–96. TP53 gene variations were found in 17% (90520). Non- synonymous TP53 mutations, found in 16.3% (85/520), were associated with older age, ducal morphology, higher grade and hormone-receptor negativity. Of the non-synonymous mutations, 12.7% (66/520) were missense and 3.6% were truncating (19/520). Only truncating mutations showed significant independently prognostic value, with an increased recurrence risk compared to patients with non-modified p53 protein (HR = 3.21, 95% CI = 1.740–5.935, p = 0.0002). p53 status had no significant predictive value for response to doxorubicin-based chemotherapy with or without docetaxel [ClinicalTrials.gov Identifier: NCT00174655].

Conclusions: p53 truncating mutations were uncommon but independently associated with poor prognosis. No significant predictive role for p53 status was detected in this patient series.

[763] Expanded Clinical and Experimental Use of SOX11 – Using a Monoclonal Antibody

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Background: The transcription factor SOX11 is of diagnostic and prognostic importance in mantle cell lymphoma (MCL) and epithelial ovarian cancer (EOC), respectively. Thus, there is an unmet clinical and experimental need for SOX11-targeting assays with low background, high specificity and robust performance in multiple applications, including immunohistochemistry (IHC-P) and flow cytometry, until now has being lacking.

Methods: We have developed SOX11-C1, a monoclonal mouse antibody targeting SOX11, and successfully evaluated its performance in western blots (WB), IHC-P, fluorescence microscopy and flow cytometry.

Results: We confirm the importance of SOX11 as a diagnostic antigen in MCL as 100% of TMA cases show bright nuclear staining, using the SOX11-C1 antibody. Furthermore, we show for the first-time that flow cytometry can be used to separate SOX11 positive and negative cell lines and primary tumors. Of note, SOX11-C1 antibody shows no nonspecific binding to primary B and T cells, can be used for analysis of B and T cell lymphomas from complex clinical samples. Dilution experiments showed that low frequencies of malignant cells (~1%) are detectable above background using SOX11 as a discriminant antigen in flow cytometry.

Conclusions: The novel monoclonal SOX11-specific antibody offers high sensitivity and improved specificity in IHC-P based detection of MCL and its expanded use in flow cytometry analysis of blood and tissue samples may allow a convenient approach to early diagnosis and follow-up of MCL patients.

[764] Molecular Characterisation of Circulating Tumor Cells in Human Metastatic Colorectal Cancer

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Introduction: Metastatic colorectal cancer (mCRC) relies on the detachment of aggressive malignant cells from the primary tumour into the bloodstream, being these Circulating Tumor Cells (CTC) the principal source of the further metastasis. Thus far, the presence of CTC is correlated with poor prognosis and there exists a clear necessity for more specific and efficient chemotherapies in the treatment of mCRC. Our aim was to overcome this adverse scenario through the massive molecular profiling of the CTC population from mCRC patients.

Material and Methods: CTCs were magnetically isolated by using anti-EpCAM coupled magnetic beads from 6 mCRC patients and 3 healthy controls and the presence of CTCs was evaluated by cytokeratins 8, 18 and 19 staining. RNA from CTCs was amplified by a whole transcriptome amplification system (WTA) and resulting material was hybridized onto gene expression arrays. Bioinformatic analyses were useful for biological interpretation of the data and RT-qPCR was used for gene expression validation. In vitro assays mimicking cellular processes related to the process of metastasis (adhesion, colony formation, anoikis . . .) and in vivo mice models of CRC metastasis functionally validated genes characterising the CTC population.

Results and Discussion: 410 transcripts were found to specifically characterise the CTC population after array signals comparison between mCRC patients and controls. Gene-gene interaction analysis generated networks related with cell migration, adhesion or apoptosis resistance, with gene ontology showing similar functions, all of them highly relevant within the expected biological role for CTCs. Signalling pathways such as RhoA, PKA, ILK, integrins or actin cytoskeleton signalling were found as relevant in CTCs. Eleven genes (TGFβ1, APP, TIMP1, CD9, CLU, ITGB5, LIMS1, RSU1, VCL, BMP6 and TLN1) were validated by real-time PCR in 20 mCRC patient and 10 control samples, showing an exceptional behaviour when used as CTC degradation biomarkers and also as prognosis indicators. This validated CTC profiling as an adequate methodology for the discovery of metastasis related markers. Knockdown of TLN1 and TIMP1 genes, relevant to key processes related with cancer spreading, resulted in an impairment of the process of metastasis. These results also demonstrated CTC profiling as a promising strategy for therapeutic target identification in metastatic disease.

Conclusions: Molecular characterisation of CTC from mCRC patients and the identification of biomarkers and therapeutic opportunities specifically targeting the CTC population should improve the efficacy in the eradication and prevention of CRC metastasis. This strategy represents an innovative and promising approach in the clinical management of mCRC patients.

[765] Blinded, Independent, Central Image Review in Oncology Trials – Will the Study Endpoint Impact the Adjudication Rate or Will the Adjudication Rate Impact the Study Endpoint?

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Background: Imaging in oncology clinical trials does not lose but rather gains increasing importance, a persisting topic of concern with a blinded, independent, central review of medical images is the adjudication rate between reviewers. With more than one reviewer it seems that an adjudication rate will simply be present, yet its mere existence can have a decisive impact on trial endpoints.

Material and Methods: Based upon our analysis of different oncology studies with various endpoints we compared the adjudication rates, how those are derived, their definition and meaning, their value and use in these clinical trials and their impact on data validity for study teams. Our analysis shows that the experience and CV-based quality of the reviewers seems not to have a major influence on the adjudication rates. However the pairing of tightly monitored reviewers can have such an impact, yet the meaning of this approach and future studies on this rate needs discussion.

Results and Conclusion: While the occurrence of adjudication does not necessarily imply that one of the reviewers made a mistake in assessing patients radiographic images, it still shows a discrepancy in opinion, in lesion selection, in tumor burden evaluation, in lesion measurement or in qualitative assessment, which leads to a discrepancy in the review results, which in turn impacts the study results. We focus with this paper on the review design and adjudication used in oncology trials with imaging endpoints based on RECIST evaluation. Since RECIST relies heavily on a quantitative assessment system measuring diameters of target lesions, whereas in parallel the qualitative component of non measured non-target lesions bears lesser importance, yet also contributes to unwanted discrepancies, together with new lesion identification. Based on FDA's recommendations and expectations, we discuss ideas for reducing the adjudication rate in future oncology trials.

[766] The Role of Beta-blockers as Antimetastatic Drugs in the Treatment of Cancer

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Introduction: Today over 90 percent of cancer-related deaths are the consequence of metastasis formation. Therefore, there is a pressing need for new antitumor drugs especially targeting metastasis formation (antimetastases). However, current antitumor drug screening assays largely aim to cytotoxicity or the inhibition of growth, and we are not able to see effects on the metastatic activity in these assays.

Material and Methods: Cell migration is an essential prerequisite for metastasis formation and may work as a surrogate for metastatic activity. We have established a three-dimensional, collagen-based assay for the investigation of tumor cell migration.

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Results: Using this assay we have shown that nopepinine led to increased migratory activity of mammary, colonic and prostate cancer cells. This effect was mediated by beta-2 adrenergic receptors; clinically established beta-blockers such as propranolol were able to inhibit this effect. These in vitro results were translated into mouse xenograft models, where nopepinine and propranolol had similar effects on the formation of lymph node metastases, but had remarkably no effect on the growth of the primary tumor. Consequently, we conducted a retrospective population study concerning the course of disease in breast cancer patients taking beta-blockers, compared to patients receiving other anti-hypertensive drugs or patients that were not hypertensive. We were the first to describe that beta-blockers reduced the risk of metastasis formation and led in consequence to a reduction in breast cancer mortality after ten years. Meanwhile, two further, independent studies concur with these results, and a similar role for beta-blockers was described in two recent studies on malignant melanoma.

Discussion: These results provide proof-of-principle that our cell migration assay can be used to investigate potential anti-metastatic drugs. Beta-blockers may constitute the first member of this new class of anticancer drugs.

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Ovarian Carcinoma Xenografts – Drug Response and Molecular Characterization

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Background: Ovarian cancer causes in western countries more deaths than any other type of female reproductive tract cancer, with estimated 15,460 deaths in the United States in 2011. The standard adjuvant therapies have greatly improved the overall survival with 70% of patients achieving complete remission after first-line platinum-based therapy. Unfortunately, almost invariably patients relapse with resistant disease. The availability of human xenografts reproducing ovarian tumors course and response to therapy would help in a better understanding of this pathology.

Material and Methods: Xenograft models: Fresh human ovarian carcinomas were subcutaneously implanted as fragments or cell suspension in six-eight weeks old female NCr-nu/nu mice (HARLAN S.p.a.). Growing tumors were serially passaged. For chemotherapeutic trials, tumor fragments were excised, minced, and suspended in sterile saline. From these the vectorcontrolwereperformedusingtheLipofectamine2000reagent.

Conclusions: These xenografts represent a useful tool not only for a better understanding of human ovarian cancer, but also for the study of the mechanisms involved in cisplatin resistance. In addition, they will be instrumental for testing new therapeutic strategies in resistant tumors.

**Chemoresistance in Breast Cancer Cells**

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Introduction: Foxo3a transcription factor is a key regulator of cell-fate decisions, such as apoptosis, cell cycle arrest and cell division. Oncogenic kinases-mediated Foxo3a phosphorylation and inactivation has been associated with tumorigenesis in breast cancer. By contrast, Survivin is an inhibitor of apoptosis protein (IAP), whose overexpression in breast cancer is correlated with poor prognosis. It has been recently shown that Survivin is a direct target for Foxo3a inhibitory transcriptional activity. This work aimed to investigate Survivin and Foxo3a role and regulation in response to doxorubicin (doxo) in breast cancer cells.

Material and Method: The human breast carcinoma cell lines MCF7 (wild-type p53) and MDA-MB-231 (mutant p53) were exposed to doxo and citoxicity was assessed through the MTT assay. Apoptosis was detected through flow cytometry DNA content analysis and Western blotting evaluation of caspases-3, -7 and -9 levels. Survivin, XIAP, p53, Foxo3a and Bim levels were assessed by Western blotting and ELISA, using the mouse-specific IgG as the Real Time PCR. Transient transfections with the Survivin-encoding plasmid and the vector control were performed using the Lipofectamine 2000 reagent.

Results and Discussion: At clinically relevant concentrations, doxo induced DNA fragmentation and reduction in caspases-3, -7 and -9 levels in a time-dependent manner in both cells, indicating that the drug could induce apoptosis independently of p53 status. Upon doxo-mediated apoptosis, Survivin levels were reduced at the protein and mRNA levels, indicating that Survivin might...
be transcriptionally inhibited. XIAP, another IAP which is stabilized by Survivin, was also downregulated after doxo treatment. When we transfected cells with a Survivin-expressing plasmid, we could observe that Survivin-overexpressing cells did not display a doxo-resistant-phenotype, indicating that Survivin downregulation is not a crucial event in doxo-induced apoptosis and suggesting that it might be the consequence of the activation of an upstream-signaling pathway. On the other side, Foxo3a total protein levels, but not mRNA levels, were increased during doxo-induced apoptosis, pointing to a post-translational modification mechanism induced by doxo treatment, which may prevent Foxo3a degradation. We also observed that the proapoptotic protein Bim—a direct Foxo3a target—were not associated with Foxo3a levels or apoptosis induction, indicating that doxo-triggered apoptotic cascade is Bim independent.

Conclusion: Our data show that Survivin overexpression cannot prevent doxo-induced apoptosis and point Foxo3a transcription factor expression as a marker for doxo cytotoxic effects in breast cancer cells. It still remains to be investigated whether Foxo3a knockdown affects Survivin expression and cell sensitivity to doxo-mediated apoptosis.

779 | An Increased CD8+ Cytotoxic T Cell Immune Response in Human Papillomavirus-related Oropharyngeal Carcinoma Correlates With Patients’ Improved Prognosis

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Background: Human papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (OSCC) defines a distinct clinical subset with improved disease-free and overall survival. HPV-positive OSCC are known to bear wild-type TP53, to express a functional p53 protein, and are therefore thought to be more chemoresistant and radioresistant. However, the molecular and cellular mechanisms that underlie the improved prognosis of HPV-positive patients are still poorly characterized.

Material and Methods: We have performed an Affymetrix GeneChip analysis by comparing the transcriptome of 11 HPV16-positive to 83 HPV-negative OSCC. The expression levels of candidate genes were measured by quantitative real-time RT-PCR on 144 RNA samples from an independent retrospective series of patients with OSCC, who received surgery followed by radio- or chemoradiotherapy. The expression of the transcripts that encode the E6/E7 viral oncogene was found in 24/144 patients. Immunohistochemistry was performed on formalin-fixed paraffin-embedded tissue. Global survival was evaluated with Kaplan–Meier and multivariable Cox regression models.

Results: Our GeneChip analysis showed that the HPV-related OSCC displayed a marked activation of signalling pathways known to play a role in adaptation and immune response. We performed a qRT-PCR evaluation of the expression of genes of these pathways in a series of 144 OSCC, and found that HPV-positive lesions expressed higher levels of CD2 (p = 0.001), Granzyme K (<CD8, <CD3) (p = 0.016) and CD4 (p = 0.032). These results were confirmed by an immunohistochemistry approach using anti-CD8-, anti-CD3, and anti-CD4 antibodies. A semi-quantitative analysis of the immunohistochemistry results showed that the stroma of HPV-related OSCC displayed an increased infiltration by CD8 lymphocytes (<p = 0.001). CD8+ and CD3+ expression correlated with improved global survival (OR = 0.22; 95% CI [0.08–0.59]; p = 0.003 and OR = 0.40; 95% CI [0.18–0.88]; p = 0.024, respectively).

Conclusions: Altogether, our results are consistent with an increased infiltration of the microenvironment of HPV-related OSCC by CD8+ T cells. This immune response seems to participate to the patients’ improved prognosis.

780 | High Nuclear Expression of Activated NF-κB is Associated With Increased Recurrence in Breast Cancer

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Introduction: Breast cancer is the most common form of cancer in women in the UK. Although therapies currently exist, they are not fully effective with many breast cancer patients developing resistance to treatment. Better understanding of the pathogenesis of breast cancer is required in order to develop novel therapeutic targets. The NF-κB pathway regulates the transcription of a wide range of genes involved in the immune response, inflammation, proliferation and apoptosis. Many of these processes are hallmarks of cancer and NF-κB has been hypothesised to be a link between inflammation and tumourigenesis. The aim of the current study is to investigate the role of the NF-κB subunit p65 in the pathogenesis and recurrence of breast cancer.

Material and Method: Immunohistochemistry was employed to assess expression levels of p65 and phosphorylated p65 at the serine 536 residue (p65 S536) in tissue samples taken from 414 breast cancer patients. Expression was quantified using the weighted histoscore technique by 2 independent observers.

Discussion: Nuclear expression of p65 S536 was observed in 81% (336/414) of patients. Those patients with tumours that expressed high levels of p65 S536 in the nuclear compartment had significantly shorter disease-free survival compared to those with low expression (6.95 years vs 7.51 years, p = 0.003). On multivariate analysis this was independent of known clinicopathological factors (p = 0.002, HR = 2.30, 95% CI: 1.35–3.92). When the cohort was subdivided, this observation was attenuated in ER positive tumours (p = 0.002) and highly proliferative tumours (high Ki67 (p < 0.001). On multivariate analysis these observations were independent of known clinicopathological factors (p = 1.5 x 10^-4, HR 4.26, 95% CI: 1.89–9.6 and p = 0.016, HR = 2.85, 95% CI: 1.21–6.71, respectively). When p65 S536 status is considered in ER positive tumours only, the effect observed on disease-free survival was negated in the tumours with low proliferation index (7.66 vs 8.32 years, p = 0.266), however was greatly potentiated in those tumours with high proliferation index (5.05 vs 8.30 years, p = 1.5 x 10^-7). These results demonstrate that the NF-κB pathway is involved in promoting recurrence in patients with ER positive, highly proliferative tumours and therefore suggest that this pathway may be involved with development of endocrine resistance and provide a novel therapeutic target. However, further investigation of p65
and other members of the pathway is required in an ER positive tamoxifen treated cohort.

Conclusion: In conclusion, activation of the canonical NF-κB pathway is associated with low disease free survival in patients with ER positive, highly proliferative tumours, suggesting that this pathway may play a role in promoting recurrence on tamoxifen.

**775 The Prognostic Impact of BRCA1 Protein Expression in Patients With Non-small Cell Lung Cancer**

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Background: BRCA1 is a 220kDa multifunctional protein which has recently gained a major scientific interest as a potential prognostic and/or predictive marker for various tumors, including non-small-cell lung cancer (NSCLC). Overexpression of BRCA1 mRNA has been associated with poor survival in chemo-naïve, stage IB and in advanced stage NSCLC patients after DNA damage chemotherapy. These findings are not confirmed by immunohistochemistry, which is more reliable and widely used tool for diagnostic purposes. On the other hand only mRNA expression does not reflect the presence of full length, functional protein. We aimed to investigate the prognostic impact of BRCA1 immunohistochemical expression in NSCLC patients, using several different antibodies.

Material and Methods: We tested five antibodies (Abcam). Three of them against different parts of BRCA1 protein: MS110 against N-terminal (1–304aa), GLK2 against C-terminal (1832–1863aa) and 17F8 against central (762–1315aa) part of BRCA1. Also, two antibodies against phosphorylated forms of BRCA1 at Ser1423 (phosphorylated during normal cellular functioning of BRCA1 and also in response to DNA damage) and Ser1524 (specifically phosphorylated by ATM in response to DNA damage). After testing several antibody retrieval methods GLK2 and 17F8 were excluded from the study. We performed BRCA1 immunohistochemistry on several tissue microarrays (TMA)s of 104 early (I, II stage) and advanced (III, IV stage) NSCLCs, using the rest of three antibodies. Patients with III and IV stage disease were treated by adjuvant cisplatin-based chemotherapy. Staining results were statistically analyzed in correlation with all available clinicopathological factors.

Results: BRCA1 MS110 staining showed extremely weak nuclear positivity in 28% of NSCLC cases, 82% were positive for Ser1423 and only 27% for Ser1524 antibody. Statistical analysis of data showed no significant correlation between BRCA1 MS110 and Ser1423 expression and given clinicopathological factors. Only BRCA1 Ser1524 nuclear positivity was significantly correlated with longer overall survival (OS) and disease free survival (DFS) in stage I and II patients (p < 0.001), whilst OS and DFS was shorter in S1524 positive stage III and IV patients (p = 0.001).

Conclusions: (1) The discrepancy between BRCA1 mRNA and protein study results might be due to the BRCA1 protein nature and inability to detect the full length, functional protein; (2) BRCA1 phosphorylation, at least in ser1524, differently determines the prognosis of early and advanced NSCLC, supposedly due to its role in DNA repair; (3) The detection of phosphorylated forms of BRCA1 might serve as useful prognostic marker for patients with NSCLC.

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**776 Copy Number Profile of Esophageal Cancer – Comparative Analysis of Adenocarcinoma and Squamous Cell Carcinoma**

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**777 Impact of BRCA2 Mutation in Primary Human Triple Negative Breast Cancer Xenografts on the Responses to Ionizing Radiation and in Vivo Tumor Growth after Treatment**

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Background: Triple negative breast cancer (TNBC) is an aggressive disease associated with a high risk of distant recurrence and poor overall survival and, as for other BC subtypes, loco-regional treatment relies on surgery and radiotherapy. To prevent relapses, there is a need for both better systemic therapies and improvements in local treatments. Primary human TNBC xenografts are a useful tool for such preclinical studies. Using two TNBC models, one of which carrying a BRCA2 mutation (De Pater et al, BJC 2010;103:1192–200), we have investigated ionizing radiation sensitivity and the impact of the treatment on the growth of irradiated tumors.

Material and Methods: Two TNBC models, the BRCA2 HBCx-17 and the wild-type HBCx-12A xenografts were subcutaneously transplanted into the flanks of nude mice. After immobilization of the animals, the tumors were locally irradiated with low energy photons generated by an X-Ray tube (voltag: 200 kV, filter: 1 mm Al + 0.3 mm Cu, mean energy: 100 keV). Irradiation fields adapted to the targeted tissue were obtained using Cerrobend shields. With a daily fraction of 3.25 Gy, the cumulative dose ranged between 0 Gy to 32.5 Gy. In addition for both models, three tumor fragments from non-irradiated tumors, 9.25 Gy-irradiated tumors with initial partial local control and subsequent progressive disease, and 19.5 Gy-irradiated tumors with initial complete remission and subsequent relapse were regrafted 3 months after treatment into nude mice. In all experiments, tumor take and growth were assessed by measuring tumor volume with a caliper twice a week for 5 months.

Results: In both TNBC models, a strong sensitivity to ionizing radiation was observed with the occurrence of complete remission seen at higher doses. However, after 32.5 Gy, this was 100% in the BRCA2-mutated xenograft and only 50% in the HBCx-12A model (p < 0.05). Similarly, relapses occurred more frequently in the wild-type xenograft (57.1% vs 14.9%; p < 0.001). With respect to the treated or untreated tumors that were regrafted into mice, no differences of tumor take, in vivo tumor growth nor kinetics were observed for the BRCA2-mutated model for the 3 tumor types, in contrast to the HBCx-12A xenograft where 9.25 Gy-irradiated tumors grew faster than the 2 other groups.

Conclusions: Our preclinical results confirm the sensitivity of TNBC to ionizing radiation and the impact of BRCA2 mutations on this sensitivity. In addition the presence of BRCA2 mutations would appear to modulate the post-radiotherapy growth of the tumors making them less aggressive. Such a characterization of highly relevant preclinical models supports their use for pharmacological assessments that will combine both radiotherapy and new therapeutic approaches to improve the outcome of TNBC patients.
Targeting Caspase-9/PP2A Interaction as a New Anti-tumor Strategy

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Background: Dysfunction in the regulation of apoptosis is associated with important pathologies, including cancers. While apoptosis is known to rely on the Bcl-2 family members and caspases, recent data suggest that serine/threonine phosphatase PP2A is a key actor in regulating both Bcl-2 family members and caspases for cell life or cell death decision. Cell penetrating peptides are molecules that can translocate into cells without causing membrane damage, leading to their proposed use as vectors for delivering therapeutic cargo. Focusing on both caspase-9 and PP2A, our goal was to develop cell penetrating peptides specifically designed against the caspase-9/PP2A interaction and evaluate their in vitro and in vivo therapeutic potential in different cancer models.

Material and Methods: We have published the Drug Phosphatase Technology (DPT) based on the design of a cell penetrating peptide obtained by association of a cell permeable shuttle, DPT-Sh1 to a sequence of interest. We have also shown that DPT can achieve a significant inhibition on an anticancer therapeutic (PP2Ac), detected by co-immunoprecipitation and confirmed by far western. We decided to generate a peptide containing the previously published penetrating sequence (DPT-Sh1) associated to the site of interaction of human caspase-9 (DPT-C9), in order to disturb the interaction caspase-9/PP2Ac. Using both mouse and human breast (BC) and non-small cell lung (NSCLC) cancer cells and tumors, we have then investigated the effect of DPT-C9 (specific to the sequence of the mouse caspase-9) and DPT-C9h (specific to the sequence of the human caspase-9). We have shown a direct interaction of human caspase-9/human PP2Ac, detected by co-immunoprecipitation and confirmed by far western. In in vivo experiments, DPT-C9 and DPT-C9h peptides were intraperitoneally administered at doses ranging from 1 to 50 mg/kg/day for up to 6 weeks.

Results: We have demonstrated that both DPT-C9 and DPT-C9h peptides specifically blocked in vitro, in vivo, and ex vivo caspase-9/PP2A interaction, and induced caspase-9 activation and apoptosis in all tested cancer cell lines. In in vivo experiments, we have showed that DPT-C9 induced significant tumor growth inhibition (TGI) in mice bearing xenogenic Polyoma Middle-T Pymt mouse breast tumors and in mice bearing Kras mutated mouse NSCLC tumors. Similarly, DPT-C9h induced significant TGI in various primary human BC and NSCLC xenografts. Finally, no toxicity has been observed in treated mice, as well as no immunogenic response in immune deficient mice.

Conclusions: We have identified an interaction between caspase-9/PP2A that may be used as a new therapeutic target. Using the cell penetrating peptide DPT-C9h blocking this interaction, we have demonstrated that DPT-C9h has an impressive in vivo and in vivo therapeutic effect and constitutes a new therapeutic approach of human cancers.

Antitumor Activity of the New Bcl-2/Bcl-xl Inhibitor S44563 in Primary Human Uveal Melanoma Xenografts

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Background: Uveal melanoma (UM) is the most common primary malignancy of the eye in adults. Although over 95% of patients have disease limited to the eye at diagnosis, 50% will develop liver metastases and ultimately die of their disease. Because of the limited efficacy of current treatments, new therapeutic strategies need therefore to be developed. Reduced capacity for apoptosis induction is one potential obstacle to therapy but, consequently, regulators of apoptosis may constitute attractive targets for anticancer therapeutics, such as the Bcl-2 family proteins. Human uveal melanomas are characterized by a high amount of Bcl-2 expression, ranging between 50% and 100% of studied cases. Such an observation has been confirmed in our panel of 16 cases. We have therefore developed preclinical orthotopic models, we have developed relevant and predictive models, we have developed preclinical orthotopic xenografts of both primary human uveal melanoma (UM) and retinoblastoma (Rb) into immune deficient mice.

Materials and Methods: Orthotopic models of human UM and Rb have been developed from two panels of subcutaneous xenografts previously established and characterized in the laboratory (Némati et al 2010; Aerts et al 2010), i.e. 6 UM models (MP34, MP41, MP42, MP55, MP65, and MM26) and 3 Rb models (RB101, RB111, and RB200). Mice bearing xenografts were sacrificed and tumors were dissected to obtain a suspension of fresh tumor cells at a concentration of 8000 cells/ml in DMEM serum-free medium. Under intraperitoneal anesthesia, 2 ml of cell suspension was injected into the subretinal space of the right eye for 3 groups of mice using a 32G needle via a Hamilton syringe. Each group was composed of 3 to 6 SCI mice. After subretinal injection, ophthalmic examination of the mice was performed weekly. When tumor cells invaded vitreal cavity and anterior chamber, the mice were sacrificed for ophthalmological pathological analyses. Finally, using the RB200 model, we have then evaluated the efficacy of 2 ultrasound intratumoral administration of bevacizumab (25 mg/kg/week for 4 weeks), melphanal (500 µg/kg/week for 4 weeks), and carboplatin (100 µg/kg/week for 4 weeks).

Results: The 6 UM cells developed in all injected eyes, 6 to 10 weeks after orthotopic transplantation. Pathological analyses confirmed UM diagnosis and conservation of histological sub-type, i.e. epithelial and/or spindle cell, than in corresponding patient’s tumors and subcutaneous xenografts. Liver tissue examination is on-going. Rb tumor cells developed in all injected eyes 4 to 6 weeks after orthotopic transplantation for RB102 and RB200. In contrast, no tumor growth was observed in injected eyes of the RB111 model. Pathological examination of the injected eyes confirmed the presence of a massive infiltration of the retina, vitreous and anterior chamber by retinoblastoma cells. Finally, we have observed a high efficacy of intracocular administration of carboplatin (17% of intracocular tumors), but not bevacizumab (100%) or melphanal (67%), in the RB200 model than in the control group.

Conclusions: We have developed relevant and available preclinical models of human UM and Rb that allow pharmacological assessment of intraocular standard therapies. We will now evaluate innovative therapeutic approaches in such a tumor situation.

Reference(s)
Androgen Receptor Phosphorylation is Associated With Clinical Outcome Measures in Hormone Naive Prostate Cancer

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Introduction: Although prostate cancer (Pca) can be readily diagnosed, estimation of likely outcome remains problematic as many will have a benign course and will not die of their disease, while some patients progress rapidly to castrate resistant disease and die within 18–24 months. Ideally, there would be tests or factors which enable the clinician to identify those patients who will die due course succumb to the disease from those who will not. In Pca activation of signal transduction cascades has been shown to alter androgen receptor (AR) activity. Although it has been suggested that changes in AR phosphorylation might be directly responsible, the candidate kinases mediating phosphorylation are not conclusive. Recent evidence suggests that Protein kinase C (PKC) phosphorylates AR at Serine 578 (pAR578).

Materials and Methods: The aim of the current study was to investigate if the relationship between AR expression and clinical prostate cancer (PCa) outcome is a direct relationship. Scansite, a predicted protein expression levels of PKC and pAR578 in a cohort of 90 patients with hormone naïve prostate adenocarcinoma.

Results and Discussion: High cyttoplasmic pAR578 expression (> median) significantly correlated with time to biochemical relapse (p = 0.034) with decreased overall survival (p = 0.034). Additionally, expression of cytoplasmic pAR578 was significantly associated with decreased disease-specific survival (p = 8.6 × 10⁻¹⁰), as was high nuclear pAR578 expression (p = 0.036). Protein expression of PKC was not significantly associated with survival. However, nuclear PKC correlated strongly with pAR578 expression, in both the cytoplasm (c.c. 0.426, p = 0.002) and nucleus (c.c. 0.469, p = 0.001).

Conclusion: These results suggest that the phosphorylation of the AR by PKC may be of functional importance in the clinical setting. Consistent with predictions by Scansite expression of PKC significantly correlated with phosphorylation of pAR578. Phosphorylation pAR578 is implicated in Pca progression and survival. Further work investigating the functional consequences of inhibition of AR phosphorylation at serine 578 is warranted.

Effect of Breast Cancer Resistance Protein (BCRP) (C421A)

Genetic Polymorphism on the Induction Response in the Treatment of Egyptian Pediatric Acute Lymphoblastic Leukemia, Children’s Cancer Hospital Egypt (CCHE)-5737 Experience

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Introduction: Although the treatment of acute lymphoblastic leukemia (ALL) is one of the success stories in pediatric oncology, but the main problem is the still developing of resistance to the structurally and functionally unrelated cytotoxic compounds which give rise to failure of treatment in 25% of patients. A variety of cellular mechanisms can give rise to maladaptive resistance due to the induction of drug resistant phenotype (MDR) which is associated with trans-membrane protein-mediated efflux of cytotoxic compounds leading to a decreased cellular drug accumulation and toxicity. Breast cancer resistance protein (BCRP) is one of the ATP-binding cassette (ABC) transporters that remove substrates out of the cell against the concentration gradient. Overexpression of BCRP leads to MDR. There were over 80 different, naturally occurring sequence variations in the BCRP gene. Actually, the most extensively studied genetic variations with potential clinical consequences of inhibition of AR phosphorylation at serine 578 is warranted.

Material and Method: Informed consent was obtained from ALL patients treated in CCHE according to modified St Jude XV study and before blood samples were obtained. Genetic polymorphism of BCRP (C421A) was done by PCR using the specific primers. The Bone marrow involvement was performed by flow cytometry.

Results and Discussion: It is the first study in the Middle East with this high number to explore this effect. We enrolled 160 newly diagnosed Egyptian ALL patients, 88 males and 72 females (age mean years). Immunophenotyping of the initial BM samples was B-precursors 83.8% versus T-cell 16.3%. The genetic polymorphism of BCRP (C421A) was (86.9%) wild type and (13.1%) mutant type. The response of patients during induction period was assessed based on BM day 15 (M1 = 80%, M2 = 16.9%, M3 = 2.5%) and MTD day 15 (<0.01% = 27%, 0.01%–0.99% = 41.3%, 1%–30% = 30%). While BM and NRD at the end of induction day 42 were (M1 = 98.8%, M2 = 1.3%) and (<0.01% = 83.3%, 0.01–0.99% = 11.9%, >1% = 3.8%) respectively. No significant correlation between genetic polymorphism of BCRP (C421A) and the BM response of patients (p = 0.246) and MTD day response rate (M = 30%) and day 42 (M = 0.964).

Conclusion: There is no significant correlation between the genetic variation of BCRP (C421A) and the response during the induction period of ALL treatment and correlation with other tran-membrane protein mediated efflux of cytotoxic agents is needed to determine the mechanism of resistance.

GSTP1 Polymorphism Can Predict Clinical Outcome in Hodgkin's Lymphoma Patients

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Introduction: Genetic polymorphism of GSTP1 could be essential in the determination of susceptibility to the toxic effects of chemicals and might also be involved in the tumor response to anticancer drugs and influence the treatment outcome. We assessed the impact of the GSTP1 A131G polymorphism on clinical outcome in patients with Hodgkin’s lymphoma (HL).

Materials and Methods: The case group comprised 97 patients with HL (mean age: 31 years, range: 17–75; 55 males and 42 females) treated in CCHE according to modified St Jude XV study and before blood samples were obtained. We investigated if the genetic polymorphism of BCRP (C421A) affects the treatment outcome. We assumed that the association of GSTP1 variant with unfavorable prognosis of HL may be attributed to a higher activity of the wild allele towards anticancer agents metabolism, as well as to its higher effect on the favouring tumor cell survival when compared with the variant allele.

Conclusions: GSTP1 polymorphism can be a useful prognostic marker in patients with HL once our data have been confirmed on a larger group of patients.

Determination of Differential Tumour Vascular Pathophysiology in Vivo by Photoacoustic Imaging

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Introduction: Angiogenesis is required for tumours to develop beyond ~1 mm³ as it delivers oxygen and nutrients to the growing tumour. It also allows potential metastatic dissemination of tumour cells. Tumour vasculature is therefore an important therapeutic target, and adequate assessment of response to vascular targeted therapy is required. Photoacoustic imaging (PAI) is a technique that has high contrast and specificity coupled with high spatial resolution. The main challenges in biological tissue is haemoglobin, and as such PAI can be used to provide structural and functional information relating to tumour blood vessel supply in a longitudinal, non-invasive manner. The aim
Functional Characterization of FXYD2 in Ovarian Clear Cell Carcinoma

M. Martinicky1, M. Zvarik2, L. Sikurova2, L. Hunakova3.

In vivo study was to use PAI to assess differing vascular pathophysiology using spectroscopic fluorescence excitation/emission matrices and concentration matrices of synchronous fluorescence spectra.

Background: Ovarian carcinoma has the first mortality rate in gynecologic malignancies. Despite the widespread use of aggressive cytoreductive surgery and the introduction of chemotherapy regimens, the overall survival has changed little over the last two decades. Ovarian clear cell carcinoma represents a remarkably distinct clinical type compared to other epithelial ovarian cancers, and carries a poorer prognosis.

Experimental procedure and Results: Using expression array analysis from 22 clear cell carcinoma tissues, FXYD2 gene is up-regulated about 4.3-fold associated with various pathological characteristics and overall survival.

Conclusion: Using expression array analysis from 22 clear cell carcinoma tissues, FXYD2 gene is up-regulated about 4.3-fold associated with various pathological characteristics and overall survival.

Fluorescence Characteristics of Urine From Ovarian Cancer Patients

D. Martinkova1, M. Zvarik1, L. Sikurova2, L. Hunakova2.

Background: Analysis of intrinsic fluorescence of human urine using fluorescence excitation/emission matrices and concentration matrices of synchronous fluorescence spectra allowed to compare healthy individuals and patients with ovarian cancer. This could contribute to a possible novel screening tool for early diagnosis of ovarian cancer.

Material and Methods: The excitation-emission matrices were scanned in the excitation wavelength range of 250–530 nm with emission taken in intervals of 270–650 nm. Synchronous fluorescence spectra were collected in the excitation wavelength range of 250–550 nm with a constant difference of 70 nm between emission and excitation wavelengths. Urine samples were examined undiluted and diluted with distilled water (1:1–1:10) in triplicate.

Results: The excitation-emission spectra revealed four distinct fluorescent bands originating from fluorescent metabolites in human urine. The synchronous fluorescence spectra were more detailed in peak resolution. We observed statistically significant difference in the intensity of peaks 330–420 nm and 370–440 nm between samples from healthy women (controls) and patients with ovarian cancer. This was also valid for the ratio of peaks 370/440 nm: 330/420 nm.

Conclusion: Based on our study, we suggest pteridines, or the whole group of pteridines, related to cellular metabolism, as suitable candidates for neoplasia-associated fluorescent markers in human urine.

Identification of Thymosin-beta-4 as Indicator for Cytotoxic Responses of Fenretinide and Vorinostat Combination Treatment

J. Koach1, O. Tan1, B.B. Cheung1, G.M. Marshall1.

Introduction: Retinoids are an important component of therapy at the stage of minimal residual disease for advanced neuroblastoma. However, 40–50% of patients treated with 13-cis-retinoic acid still relapse, indicating necessity for more effective retinoid therapy. Fenretinide (4-HPR) and vorinostat also known as suberoylanilide hydroxamic acid (SAHA), were utilised in early phase single agent paediatric oncology trials, but only stabilised disease.

Material and Methods: We used human neuroblastoma and medulloblastoma cell lines, flow cytometry, gene-expression analyses, siRNA knockdown and xenograft tumour models to evaluate 4-HPR + SAHA combination treatment for therapeutic synergy and biomarkers of response.

Results and Discussion: At clinically relevant concentrations of 4-HPR (1.33–3 μM) + SAHA (0.22–0.5 μM) the combination therapy exerted potent cytotoxic effects in multiple neuroblastoma and medulloblastoma cell lines (combination index < 1). The proportion of apoptotic cells was markedly increased in neuroblastoma cells compared with non-malignant MRC-5 cells. The cytotoxic effect of 4-HPR + SAHA was much greater than 13-cis-retinoic acid + SAHA. In vivo xenograft experiments of BE(2)-C cells treated with the flank of athymic nude mice treated with 4-HPR (1.45 mg/kg, i.v.) + SAHA (35 mg/kg, i.p.) also demonstrated therapeutic synergy. To identify biomarkers of sensitivity to 4-HPR + SAHA, we evaluated the transcriptional changes in neuroblastoma cells treated with the combination. The four candidate biomarker genes for which changes in expression level best correlated with the cytotoxic responses were RARα, RARγ, Thymosin-beta-4 (Tβ4), and Systostatin-3 (SYNGR3). We performed siRNA knockdown of Tβ4 in neuroblastoma cells treated with 4-HPR + SAHA and found treatment synergy was lost without Tβ4 expression, indicating Tβ4 was necessary for the 4-HPR + SAHA cytotoxic effect.

Conclusion: Our data suggest that 4-HPR + SAHA is an effective alternative combination therapy in vitro and in vivo in neuroblastoma and medulloblastoma.

The Presence of Circulating Tumour Cells is a Negative Prognostic Factor in Lung Cancer Patients

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Background: We tested the hypothesis that the presence of circulating tumour cells (CTCs) is a negative prognostic factor in patients with lung cancer. Lung cancer is one of the leading causes of cancer death and has become an increasingly urgent worldwide health problem. Assessment of the CTCs in patients with this highly malignant disease could prevent aggressive surgery in patients with metastatic spread.

Material and Methods: This was a prospective study to test the presence of CTCs in peripheral blood, pulmonary blood and bone marrow in 108 lung cancer patients (69 males, 39 females; mean age 66.2 years, ranging from 29 to 82 years) at the time of surgery using real-time RT-PCR for carcinoembryonic antigen (CEA), C-reactive protein (CRP), Epstein-Barr virus (EBV) and human cytomegalovirus (CMV) in peripheral blood. The presence of CTCs was examined by immunocytochemistry with monoclonal antibodies specific for the human D2-40 antigen.

Results: 62 lung cancer patients followed up for more than 1 year were involved into the overall survival analysis. 27 of them (32.9%) died, the overall survival median was 19.6 months.

Conclusion: A significant association between C-met expression level in the pulmonary blood and clinical stage (p < 0.044) and lymph node involvement (p < 0.0023) was found. C-met marker also showed significantly higher positivity ratio in the peripheral (p < 0.034) and pulmonary (p < 0.04) blood in patients with
The lung cancer patients with the presence of CTCs in the peripheral blood showed a higher hazard ratio (p = 0.69) compared to patients without CTCs in the peripheral blood (HR = 0.2cm3, RA = 1.38 ± 0.35cm3, THAL = 1.43 ± 0.23cm3, RA + THAL = 1.5 ± 0.27cm3). These results are in line with our previous findings of the work to identify predictive molecular markers for patients’ selection for standard chemotherapies, 5-fluorouracil alone. Nevertheless, the outcome of patients with resected pancreatic cancer in a randomized trial. The main aim of the study was to identify predictive molecular markers for patients’ selection for chemoradiotherapy with interferon-alpha. Methods: RNA from the frozen tissue samples of patients with higher and lower overall survival (OS) was isolated and used for gene expression profiling with Illumina technology. Bioinformatics was applied to select differentially expressed genes. The selected gene candidates were further validated by Real-Time PCR. Survival analysis with gene candidates as predictors was applied to find predictive markers with respect to OS and disease-free survival (DFS). Results: 12 gene candidates were selected from the Illumina array data as potential candidates in lung cancer patient dataset with the use of bioinformatic tools. Expression level of these genes was measured with Real-Time PCR. Afterward we applied the Cox Proportional Hazard model on the selected 12 gene candidates to identify the significant predictors of the OS and disease-free survival (DFS). High significant predictors for the OS of the patients appeared to be: GAGES (p-value = 0.0099), as well as MAP3K2 (p-value = 0.055) and RET1L (p-value = 0.06). 3 genes were detected to be highly significant predictors for the DFS: GAGES (p-value = 0.0088), MAP3K2 (p-value = 0.02444) and TCEA1 (p-value = 0.0034). Conclusion: Expression level of GAGES, MAP3K2 and RET1L has been found to be predictive for the OS, and GAGES, MAP3K2 and TCEA1 for the DFS of patients undergoing chemoradiotherapy. These markers should be prospectively evaluated in a future clinical trial.
and relatively short patient survival, new therapeutic strategies are required. Despite the promising introduction of the proteasome inhibitor bortezomib in the clinic, disease control and relapsed features occur after initial response. When comparing the behavior of both bortezomib-resistant and bortezomib-sensitive cell lines in a xenotransplant model, we observed an increased tumorigenicity of bortezomib-resistant cells in vivo, suggesting a major capacity of these tumors to interact with lymphoid microenvironment. As the immunomodulatory drug lenalidomide has been shown to modulate tumor-stroma interaction in several B cell malignancies, we assessed the activity in vitro and in vivo of this agent either alone or combined with the proteasome inhibitor in both bortezomib-resistant and bortezomib-sensitive samples. Lenalidomide single agent was found to exert modest antitumoral activity in 210 MCL cell lines, corresponding to those cells with either primary or acquired resistance to the proteasome inhibitor. Conversely, mice bearing bortezomib-resistant tumors and treated for 3 weeks with a 10−50 mg/kg/day regimen of lenalidomide, showed a 30 to 45% reduction in tumor burden when compared to vehicle-treated group (p < 0.05).

The corresponding biopsies harbored several hallmarks of lenalidomide activity in malignant B cells such as CD80 and CD40L upregulation, together with a remarkable decrease in mitotic index, c-myc down-regulation, p27 cytosolic accumulation and caspase-3 processing. Similarly, bortezomib-resistant MCL cell lines treated for 72h with 1 μM lenalidomide showed lower c-myc levels, as well as p27 accumulation, caspase-3/7 activity and apparition of hypodiploid cells. When combined to bortezomib therapy (0.15 mg/kg, twice a week), lenalidomide induced a 37% and a 66% inhibition of tumor growth when compared to lenalidomide and vehicle groups, respectively (p = 0.02). In accordance, lenalidomide showed synergistic effect in vitro with bortezomib in co-culture system associating the MCL cell line Jeko-1 to the dendritic-like cells BDCM, by modifying the secretion pattern of these latest.

Together, these results suggest that single agent lenalidomide is preferentially effective in MCL cases resistant to bortezomib, by targeting c-myc-driven tumorigenesis. Additionally, lenalidomide may overcome the protection offered by lymphoid tumor microenvironment toward bortezomib treatment, thus warranting a promising clinical activity of lenalidomide-bortezomib combination in MCL cases refractory to bortezomib.

**Material and Methods:** We have generated ‘knock-in’ mouse strains expressing various levels of the R246S mutant p53 (equivalent to the human R249S mutant), to study mutant p53 properties during acute p53 activation in the absence of MDM2, unlike the R172H mutant, highlighting specificity of the dominant-negative (DN) effect, or exert gain-of-function (GOF) properties upon loss of the other wild-type allele. However, the specific roles of mutant p53 have been extensively studied in different tumor types, regardless of their differentiation state. Threshold levels of mutant p53expression vary in different cell types, leading to the observation that the initial p53 response after damage is not relevant for tumor suppression. Furthermore, the R246S mutant did not promote tumorigenesis compared to p53−/− mice in IR or oxygenated-tumored mice, even in the absence of MDM2, unlike the R172H mutant, highlighting specificity of mutant p53 in promoting GOF.

**Conclusion:** Together, these data demonstrate that mutant p53’s DN property only affects acute responses, whereas GOF is not universal, being cell-type specific.
The Novel PI3K Kinase Inhibitor NVP-BKM120 Shows in Vitro and in Vivo Efficacy in Follicular Lymphoma by Disrupting Microenvironmental Signaling

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Follicular lymphoma (FL) is the second most common B-non Hodgkin Lymphoma characterized by the t(14;18)(q32;q21) leading to over expression of the anti-apoptotic Bcl-2. It is now accepted that FL pathogenesis may be the result of a functional cross talk between the effects of this genetic alteration and the influence of its immune microenvironment, mainly in the bone marrow (BM) and lymph nodes (LN). Although FL is an indolent tumor, up to one-third of cases can progress to a more aggressive and usually fatal disease, leading to short survival. The immune microenvironment in the LN plays an important role in tumor development. Two outcome-related signatures have been identified: IR1 (genes expressed by accompanying T-cells, associated with better outcome) and IR2 (genes expressed by macrophages and Follicular Dendritic Cells (FDC), associated with inferior survival). In the recent years PI3K is one of the most attractive target in cancer therapy. In FL PI3/4 kinase pathway is constitutively activated as a consequence of survival signals coming from tumor microenvironment through cytokine/chemokine secretion and ligand-receptor interactions that include B-cell receptor (BCR). In this context, we have evaluated the potential interest of the novel and specific PI3K inhibitor NVP-BKM120 (Novartis) as a new therapy in FL. We found that NVP-BKM120 induces variable cytotoxic and cytostatic effect in FL cell lines, and limited toxicity in FL primary samples (10-20%) in accordance with other specific PI3K inhibitor WZC170). NVP-BKM120 efficiently blocks constitutive activation of PI3K/AKT pathway in FL cell lines, and completely abrogates AKT activation derived from co-culture with BM stromal cells or FDCs or consequent to BCR ligation. NVP-BKM120 also showed in vivo efficacy in a xenograft mouse model reducing tumor size by 35%. Moreover, immunohistochemistry analysis revealed a marked decrease in the expression of pAKT and its downstream target pS6rp in tumors from NVP-BKM120-treated mice. These results warrant further studies of NVP-BKM120 in FL. We are now trying to increase its therapeutic index by combination with different agents such as the B3H-mimetic ABT-263 which is able to antagonize the over expression of Bcl-2, not affected by NVP-BKM120 action, or anti-CD20 antibodies, commonly present in FL current regimens.

Mammalian Target of Rapamycin (mTOR) Activity as a Potential Target in Human Diffuse Large B-cell Lymphomas and Hodgkin Lymphomas

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Background: PI3K/AKT/mTOR signaling output is elevated in a large fraction of human neoplasms. This increased activity could be associated with lower mTORC1 and mTORC2 activity, as a consequence of survival signals coming from tumor microenvironment through cytokine/chemokine secretion and ligand-receptor interactions that include B-cell receptor (BCR). In this context, we have evaluated the potential interest of the novel and specific PI3K inhibitor NVP-BKM120 (Novartis) as a new therapy in FL. We found that NVP-BKM120 induces variable cytotoxic and cytostatic effect in FL cell lines, and limited toxicity in FL primary samples (10-20%) in accordance with other specific PI3K inhibitor WZC170). NVP-BKM120 efficiently blocks constitutive activation of PI3K/AKT pathway in FL cell lines, and completely abrogates AKT activation derived from co-culture with BM stromal cells or FDCs or consequent to BCR ligation. NVP-BKM120 also showed in vivo efficacy in a xenograft mouse model reducing tumor size by 35%. Moreover, immunohistochemistry analysis revealed a marked decrease in the expression of pAKT and its downstream target pS6rp in tumors from NVP-BKM120-treated mice. These results warrant further studies of NVP-BKM120 in FL. We are now trying to increase its therapeutic index by combination with different agents such as the B3H-mimetic ABT-263 which is able to antagonize the over expression of Bcl-2, not affected by NVP-BKM120 action, or anti-CD20 antibodies, commonly present in FL current regimens.

mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis. mTOR inhibition may slow down progression in heavily treated patients and additional treatment, especially in patients with relapse and/or poor prognosis.
Role of HERG1 Potassium Channel in Both Malignant Circulating Tumor Cells After Neoadjuvant Chemotherapy Predict Frequent Aberrant Expression of the Human Ether a Go-go Integrative Marker Analysis and Risk Assessment in Stage II HNSCC Tumors

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Introduction: Evidences indicate that HERG1 voltage-gated potassium channels could represent new valuable membrane therapeutic targets and diagnostic/prognostic biomarkers in various cancers. This study is the first to investigate the expression pattern of HERG1 potassium channel subunit in both primary tumors and precancerosis lesions to establish its clinical and biological role during the development and progression of head and neck squamous cell carcinomas.

Material and Method: HERG1 protein expression was evaluated by immunohistochemistry in paraffin-embedded tissue specimens from 133 patients with laryngeal and oral squamous cell carcinomas and 75 patients with laryngeal dysplasia, and correlated with clinical data. In vitro functional studies in HNSCC-derived cell lines further contributed to clarify the pathobiological role of HERG1 potassium channel subunit.

Results and Discussion: Our findings demonstrate that HERG1 is frequently aberrantly expressed in a high percentage of primary tumors (87%), whereas expression was negligible in both stromal cells and normal-adjacent epithelia. HERG1 expression increased during head and neck squamous cell carcinoma progression and was significantly associated with lymph node metastasis (P = 0.04), advanced disease stages (P < 0.001), regional tumor recurrence (P = 0.004), distant metastasis (P = 0.03), and reduced disease-specific survival (P = 0.012, log-rank test). HERG1-positive expression was also detected in 31 (41%) of 75 laryngeal dysplasias. Interestingly, HERG1 expression increased with the grade of dysplasia; however, HERG1 expression but not histology correlated significantly with increased laryngeal cancer risk (P = 0.007). In addition, functional studies in head and neck squamous cell carcinoma-derived cell lines further revealed that HERG1 expression promotes anchorage-independent and -dependent cell growth and invasive capability, although independently of its ion-conducting function.

Conclusion: Our data demonstrate that HERG1 expression is a biological and clinical relevant feature in head and neck squamous cell carcinoma progression and also during malignant transformation and a promising candidate marker and therapeutic target for head and neck squamous cell carcinoma prevention and treatment.

Frequent Aberrant Expression of the Human Ether a Go-go (hEAG1) Potassium Channel in Head and Neck Cancer – Pathobiological Mechanisms and Clinical Implications

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Introduction: Compelling evidence indicates that the human ether-a-go-go voltage-gated potassium channels (hEAG1) may represent new valuable membrane therapeutic targets and diagnostic/prognostic biomarkers in various cancers. This study is the first to investigate the expression of hEAG1 potassium channel subunit in both primary tumors and HNSCC-derived cell lines as well as to explore its clinical and biological role in the progression of head and neck squamous cell carcinomas.

Material and Method: hEAG1 mRNA expression was analyzed by QRT-PCR in a prospective series of 54 fresh HNSCC tissue specimens and also epigenetic transcriptional regulatory mechanisms (DNA methylation by pyrosequencing and histone acetylation by chromatin immunoprecipitation).

Results and Discussion: Our findings demonstrate that hEAG1 is frequently aberrantly expressed in a high percentage of primary tumors (83%, 45/54 cases) and HNSCC-derived cell lines (83%, 10/12 cell lines). hEAG1 expression increased during HNSCC progression and was more frequent in advanced tumors. Strikingly, hEAG1 expression was also detected in a notable proportion (39%, 17/44 cases) of patient-matched normal adjacent mucosa, whereas no expression was detected in normal epithelia from non-oncologic patients without exposure to tobacco carcinogens. In an attempt to identify the underlying mechanisms of aberrant hEAG1 expression in HNSCC, we found that hEAG1 gene copy gain occurred at a low frequency (15%, 13/88 cases) in primary tumors but was not observed in early stages of HNSCC tumorigenesis. Furthermore, this study provides original evidence supporting the involvement of histone acetylation (i.e. H3Ac and H4K16Ac acetylation marks) in the regulation of hEAG1 expression in HNSCC. In addition, functional studies in HNSCC cells further revealed that hEAG1 expression is a biologically relevant feature that promotes cell proliferation and invasion, although independently of its ion-conducting function.

Conclusion: Our findings strongly support the notion that hEAG1 may represent a promising candidate as tumor marker and membrane therapeutic target for HNSCC treatment.

Circulating Tumor Cells After Neoadjuvant Chemotherapy Predict Survival in Non-metastatic Breast Cancer

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Introduction: Circulating tumor cells (CTCs) predict outcome in metastatic breast cancer, but their significance is unclear in non-metastatic patients. Furthermore, it is unclear if the presence of CTCs after completion of neoadjuvant chemotherapy (NACT) predicts worse outcome. The purpose of this study was to determine if CTCs after NACT predict worse outcome.

Methods: Clinical stage I-II breast cancer patients seen at a single tertiary cancer center provided informed consent to participate in an IRB-approved study involving collection of blood (7.5 ml x 2 tubes) at the time of surgery for their primary breast cancer. CTCs were detected using the CellSearch® system. A positive result was defined as the presence of one or more cells per 7.5 ml blood since the threshold for positivity has not been established in non-metastatic breast cancer. Statistical analyses used chi-square and Fisher’s exact test.

Results: One hundred and thirty seven patients were prospectively enrolled. Median age was 52 years and median follow-up was 34 months. Nine percent of patients had T1 disease, 36% T2, 20% T3, and 35% T4. Fifty-four percent of patients (73/137) had ER positive and 38% (52/137) had PR positive disease. Thirty percent of patients (41/137) were HER-2 positive. Twenty-eight percent (38/137) had triple-negative tumors. Eighty-six percent (93/137) had lyme node positive disease. One CTC was found in 27% (37/137) of patients post-NACT, but its presence did not predict worse outcome (p = NS). Two or more CTCs were present in 9% (12/137) of patients. Of the 20 patients who relapsed, 6 had 2 or more CTCs (P = 0.002), while of the 14 patients who died, 4 had 2 or more CTCs (P = 0.001).

Conclusions: Presence of two or more CTCs after NACT predicted worse relapse-free and overall survival in patients with stage I-II breast cancer.

Integrative Marker Analysis and Risk Assessment in Stage II Colon Cancer


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Background: Colorectal cancer progression is governed by genetic and epigenetic alterations that can occur in multiple parallel pathways. However, with the exception of KRAS mutations, molecular markers currently play no role in risk stratification and therapy decision. E.g., risk assessment based on clinico-pathological parameters is not reliable for patients with stage II colon cancer (locally restricted disease). We hypothesize that the individual recurrence risk is defined by tumor genetics, and have therefore analyzed a panel of molecular genetic markers for risk prediction.

Material and Methods: Normal and microdissected fresh frozen tissue from 232 patients (T3-4 N0 M0) with complete tumor resection and median follow-up of 97 months was analyzed for microsatellite stability by multiplex PCR, KRAS exon 2, and BRAF exon 15 mutations by high-resolution melting analysis. Moreover, gene expression of the WNT/pancreatic surrogate marker osteopontin and the metastasis-associated genes SASH1 and MACC1 was determined for 179 patients. The results were correlated with metastatic disease as the primary endpoint (n = 22 patients).

Results: Mutations of KRAS were detected in 30% of patients, mutations of BRAF in 15%, and microsatellite instability in 26%, in good accordance with reported findings. Risk of recurrence was associated with KRAS mutation.
Dual MTORC1/2 and HER2 Blockade Results in Antitumor Activity

Transmodulation of ErbB Receptors by Proinflammatory Mediator

Results:

Inhibition of HER2/HER3 phosphorylation, PI3K/Akt/mTOR and ERK signaling pathways was evaluated by western blot. Tumor growth inhibition following treatment with lapatinib, INK-128 (a dual mTORC1/2 inhibitor) or the combination of both agents was evaluated in three different animal models: two cell-based xenograft models refractory to both trastuzumab and lapatinib, and a xenograft derived from a patient who relapsed on trastuzumab-based therapy.

Results: The addition of lapatinib to INK-128 prevented both HER2 and HER3 phosphorylation, resulting in inhibition of both PI3K/Akt/mTOR and ERK pathways. This dual blockade produced synergistic induction of cell death in five different HER2 positive cell lines resistant to trastuzumab and lapatinib. In vivo, both cell line-based and patient-derived xenografts showed exquisite sensitivity to the antitumor activity of the combination of lapatinib and INK-128, which resulted in durable tumor shrinkage and exhibited no signs of toxicity in these models.

Conclusions: The simultaneous blockade of both PI3K/Akt/mTOR and ERK pathways used as a therapeutic approach for cancer treatment.

Monitoring of Human Lung Tumour Progression Inhibited by TPFI-2 Using Imaging in Mice Orthotopic Models

Background:

Lung cancer is the main cause of cancer deaths worldwide. The major types of lung cancer exhibiting specific biological features are non-small cell lung cancer (NSCLC), 80% of cases, and small cell lung cancer (SCLC), the most aggressive that affects 15% of patients.

In this study, we developed clinically relevant nude mouse orthotopic models of SCLC and NSCLC that are reliable and mimic various stages of lung cancer. Moreover, bioluminescence imaging (BLI) combined with computed tomography (CT) allowed a longitudinal monitoring of tumour growth in living mice. These models could be used to evaluate the molecular aspects of tumour progression and herein the impact of the Tissue Factor Pathway Inhibitor-2 (TFPI-2) that inhibits plasmin and matrix metalloproteinases involved in tumour invasion.

Material and Methods: NC/Nga2 mice from SCLC were modified to express firefly luciferase and passaged twice subcutaneously to enhance tumourigenesis. This xenograft was then transplanted and stable clones expressing high or low levels of TFPI-2 were selected. Stable TFPI-2 down-regulation in the NCI-H460 NSCLC line was obtained using specific micro interfering RNA (miRNAs). Cells resuspended in EDTA (with/without Matrigel) RPMI medium containing a 99mTc-labelled colloid were implanted intrabronchially with a catheter inserted into the trachea and positioned into the main bronchus using X-ray-guided imaging. Cell depletion of lung was assessed by scintigraphy. Tumour progression was followed by BLI and CT imaging and results were confirmed by histological analyses.

Results: When NC/Nga2 cells were implanted with EDTA, there was no bioluminescence from the lung. However, histopathology analysis revealed small nodules invading the lung alveoli, as can be observed in human SCLC when diagnosed early. Multiple tumour nodules were observed in 60% of animals implanted with cells in Matrigel medium. However, a strong bioluminescence activity was located in the lung in 80% of mice when both EDTA and Matrigel were used (Iochmann et al. 2012). With NCI-H460, tumours located in the lower and middle parts of the lung were observed with CT in 95% of mice after a 4-week period. Histological analyses showed tumours with well-delineated borders as observed in human NSCLC. Monitoring of lung tumour growth using BLI in combination with measurement of tumour volume with CT and histology analysis have been further used to study the impact of a tumour suppressor gene, the TPFI-2, on tumour progression. We demonstrated that TPFI-2 down-regulation in cancer cells such as in NCI-H460 miRNA promoted non-small lung cancer progression (Gaud et al. 2011). In contrast, TPFI-2 expression in NCI-H205 reduced tumour growth.

Conclusions: Our nude mice orthotopic model resembles various stages human lung cancer and then could be useful for evaluating new therapeutic strategies.

Transmodulation of ErB Receptors by Proinflammatory Mediator

Substance P

Background:

Inflammation is a hallmark of cancer. Many tumors with inflammatory components are driven by TFPI-2 down-regulation in cancer cells such as in NCI-H460 miRNA promoted non-small lung cancer progression (Gaud et al. 2011). In contrast, TPFI-2 expression in NCI-H205 reduced tumour growth.

Conclusions:

Our nude mice orthotopic model resembles various stages human lung cancer and then could be useful for evaluating new therapeutic strategies.
Experimental Therapeutic Approach of Orthotopic and Subcutaneous Human Retinoblastoma Xenografts for the Human Anti-VEGF Antibody Bevacizumab, Alone or in Combination With Carboplatin

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Introduction: Treatment of retinoblastoma (RB) could require enucleation in unfavorable disease. In order to assess innovative therapeutic approaches of RB, we have developed preclinical orthotopic and subcutaneous human RB xenografts into immunodeficient mice.

Material and Methods: Three subcutaneous RB models (RB102, RB111, and RB200) have previously been developed and characteIZED (Aerts et al, Photodiagnosis Photodyn Ther 2010;7:275-83). The different in vivo treatments were: (10 mice/group): Isotype control antibody (15 mg/kg twice a week, 4 weeks), bevacizumab (15 mg/kg Twice a week, 4 weeks), and Rb200. bevacizumab (doses as mentioned above). Tumor growth was then assessed by measuring the tumor volumes and mice were sacrificed when their tumor reached a volume of 2500 mm3. Orthotopic models were treated from subcutaneous RB xenografts. Mice bearing xenografts were sacrificed and tumors were dissected to obtain a suspension of fresh tumor cells at a concentration of 8000 cells/ml in DMEM serum-free medium. Under intraperitoneal anesthesia, 2 ml of cell suspension was injected into the subretinal space of the right eye for 3 groups of mice using a 32G needle via a Hamilton syringe. Using the RB200 model, we have evaluated the efficacy of 2 μl intracocular administration of bevacizumab (25 mg/kg/week for 4 weeks) or carboplatin (100 μg/kg/week for 4 weeks). After sub-retinal injection, ophthalmic examination of the mice was performed weekly. When tumors invaded vitreal cavity and anterior chamber, the mice were sacrificed.

Results: When administerd alone, bevacizumab induced a tumor growth inhibition (TGI) of 63%, 40%, and 28% in the RB102, RB111, and RB200, respectively. Similarly, carboplatin alone induced a TGI of 72%, 0%, and 33% in the RB102, RB111, and RB200, respectively. In contrast, when bevacizumab and carboplatin were combined, we observed a TGI of 98%, 53%, and 78% in the RB102, RB111, and RB200, respectively. In the orthotopic RB200 model, we have observed a high efficacy of intracocular administration of carboplatin (17% of intracocular tumors), but not bevacizumab (100%). Finally, assessment of efficacy between subcutaneous and orthotopic transplanted RB200 model was concordant for both bevacizumab and carboplatin.

Conclusion: As bevacizumab and carboplatin were not highly efficient when administerd alone, their combination induced a significantly better TGI in the 3 tested RB models. Moreover, we have developped relevant and available preclinical models of human RB that allow pharmacological assessment of intraocular standard therapies.

In vivo Diagnostic and Therapeutic Methods for Photodynamic Therapy

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Introduction: Photodynamic therapy (PDT) is a minimally invasive modality approved for clinical treatment for several types of cancer. In PDT, a photosensitizer (PS) is accumulated in targeted tissue, then is activated by a light of specific wavelength. The optimal PDL of light needed to cause photoconversion is characteristic for each PS and determined by its spectral properties. Anaerobic tumors can present a significantly different oxygizonation from well-oxygenated tissues.

Results: When administerd alone, bevacizumab induced a tumor growth inhibition (TGI) of 63%, 40%, and 28% in the RB102, RB111, and RB200, respectively. Similarly, carboplatin alone induced a TGI of 72%, 0%, and 33% in the RB102, RB111, and RB200, respectively. In contrast, when bevacizumab and carboplatin were combined, we observed a TGI of 98%, 53%, and 78% in the RB102, RB111, and RB200, respectively. In the orthotopic RB200 model, we have observed a high efficacy of intracocular administration of carboplatin (17% of intracocular tumors), but not bevacizumab (100%). Finally, assessment of efficacy between subcutaneous and orthotopic transplanted RB200 model was concordant for both bevacizumab and carboplatin.

Conclusion: As bevacizumab and carboplatin were not highly efficient when administerd alone, their combination induced a significantly better TGI in the 3 tested RB models. Moreover, we have developped relevant and available preclinical models of human RB that allow pharmacological assessment of intraocular standard therapies.

Non-invasive Prognostic Tools for Phototherapeutic Response in Murine Tumors

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Material and Method: Model: S-91 melanoma and Lewis Lung Carcinoma tumors were grown in of DBA/2 and C57 mice. Experiments were approve by the Committee for Ethics of Experiments on Animals (permission no. 114/96). In PDT: The bacteriotoxin (2 mg/kg) were administrated intravenously into DBA/2 and C57. 15 min – 72 h later tumors were irradiated with 100 J/cm2 of light at λ=750 nm.

Conclusion: To our knowledge, this is the first time that a system for monitoring chlorophyll fluorescence in vivo for phototherapeutic response to treatment depends on: oxygen level, vasculature physiology, concentration of PS and light doses. To provide therapeutic success, total pressure of oxygen (pO2) and blood perfusion measurements were performed using Electron Paramagnetic Resonance (EPR) and Laser Doppler Flowmetry (LDF). To test the efficacy of PS before injection, the PS fluorescence (F) measurements in vivo were performed. All tested models are non-invasive, direct and quantitative.

[Poster Sessions]

able to induce cell death in gynecologic epithelial cancer cells. Interestingly, retrospective evidence suggests that those obese women concurrently using statins, as treatment of hypercholesterolemia, during their cancer treatment experienced better survival than those non-users. In the present work we investigate the effects of statins in leptin-induced signaling, migration and invasiveness in epithelial ovarian cancer cells.

Material and Methods: Upon mock or Simvastatin (1 μM for 24 h) pre-incubation, three human ovarian cancer cell lines (A2780, SKOV3, HEY) and primary tissue culture cells (established from advanced epithelial ovarian cancer) were incubated with leptin (100 ng/ml, up to 24 h). Under these conditions, MTS, matrigel invasion, and wound healing assays were conducted. Immunoblotting for key players in leptin signaling pathway (pSTAT3, pERK, pAKT, RhoA, pFAK) upon similar conditions, with or without adding specific inhibitors (anti-leptin receptor antibody, AG490, LY and fasudil) were also carried out.

Results: Leptin increases migration and invasiveness of ovarian cancer cells through leptin receptor-mediated activation of JAK/STAT3, PI3K/AKT, and RhoA pathways, since anti-leptin receptor antibody, AG490, LY and fasudil, respectively, all significantly decrease leptin-mediated cell migration and invasion. Simvastatin pre-treatment significantly reduces leptin-induced cell migration and invasion of ovarian cancer cells. As observed with specific inhibitors, immunoblotting shows that Simvastatin pre-treatment decreases leptin-mediated activation of all key players involved in leptin signaling pathway and participating in migration and invasiveness of cancer cells.

Discussion: Leptin-mediated effects in migration and invasiveness of cancer cells offer a possible explanation for the more aggressive behavior of ovarian cancer and the lower survival observed in obese patients. The inhibitory effect of statins in leptin-induced signaling (besides its dose-dependent apoptotic effect in ovarian cancer cells) justifies considering the inclusion of lipophilic statins in the design of future clinical trials.

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Development of a Fast in Vitro Assay to Screen New Gene Expression of Carbonic Anhydrase IX in N-diethylnitrosamine-induced, the most frequent histological RCC subtype in humans. Carbonic anhydrase IX (CAIX) is a potassium channel in the sinusoidal membrane of which have already been implicated in the pathogenesis of colon cancer, high expression in different stages of pancreatic cancer, and within a 5-day culture period, the exocrine cells derived from KC and within a 5-day culture period, the exocrine cells derived from KC and PCC mice displayed a progressive increase in CAIX expression over time.

Conclusions: Our assay offers a rapid and reliable method to assess the expression of CAIX in pancreatic cancer cell lines and primary cultures, allowing a quick screening of new candidate molecules that could be potential targets for the treatment of this deadly tumor.
Conclusion: CAX1 expression increases through carcinogenesis model since early stages and during tumor progression which suggests that CAX1 may play a significant role in tumor evolution. Abatement of CAX expression by TSE may represent a mechanism by which TSE contributed to the renal cancer incidence reduction previously observed.


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Background: We previously identified amplified fibroblast growth factor 1 (FGFR1) as a therapeutic target for small molecule inhibitor therapy in squamous cell lung cancer (L-SCC), resulting in currently running clinical trials treating patients with stage III disease. As most patients present with metastatic stage of disease, we attempt to demonstrate FGFR1 amplification in lymph node metastases of amplified primary tumors. Our study aims to give a rational to include these patients in a targeted small molecule inhibitor therapy.

Methods: We assessed 75 formalin-fixed paraffin-embedded (FFPE) primary L-SCC samples. Of these, 46 samples were primary tumors with corresponding FFPE lymph node metastasis, which were also assessed. The biotin-labeled FGFR1 target probe spanning the 8p11.23 to 8p11.12 locus was used to determine the FGFR1 amplification status using fluorescence in situ hybridization (FISH).

Results: Of 39 assessable primary L-SCC with corresponding lymph node metastasis, 7 samples displayed FGFR1 amplification (18%). All of these primary tumors also harbored FGFR1 amplification in their lymph node metastasis. Non-amplified tumors never displayed FGFR1 amplification in their corresponding metastases.

Conclusion: We found FGFR1 amplification not only in primary L-SCC, but also in corresponding lymph node metastasis, suggesting that this genetic aberration is a clonal event in tumor genesis. Furthermore, our study provides data indicating new therapeutic possibilities for patients suffering not only primary, but also metastatic FGFR1 amplified SCC lung cancer disease.


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Background: Castration resistant prostate cancer (CR-PCa) is the most aggressive form of prostate cancer (PCa) having a poor prognosis, and is a significant therapeutic challenge. The key to the development of novel therapeutic targets for CR-PCa is to decipher the molecular alterations underlying this lethal disease. The aim of our study was to perform whole exome sequencing and gene copy number analysis on 5 CR-PCa/normal paired formalin fixed paraffin embedded (FFPE) samples using the SOLiD4 next generation sequencing platform.

Materials and Methods: Genomic DNA was extracted from 5 CR-PCa/normal paired FFPE samples. The DNA was subjected to targeted exon capture using the Agilent SureSelect kit. The captured DNA was sequenced using the SOLiD4 next generation sequencing platform. The sequencing output was mapped, sorted, filtered and annotated using well-known human genome databases. The results were further analyzed for SNPs and copy number variations. A set of amplified/deleted genes were validated using fluorescence in-situ hybridization (FISH) assays with a PCa progression cohort.

Results: Whole exome sequencing analysis identified focal regions of deletion, which included well-known tumor suppressors such as NKX3.1 and PTEN. Focal regions of amplification included well-known genes such as CMYC and AR that are known to play a role in PCa. Furthermore, we identified several amplified genes as druggable targets e.g. HDAC6, NTRK1, PLD1, SPHK1, and SIRT7. NTRK1 is a kinase that plays an active role in cell proliferation. HDAC6, PLD1, SPHK1 and SIRT7 regulate numerous complex cellular processes including signal transduction, transcription and apoptosis.

Conclusions: This is the first study to use whole exome sequencing approaches on FFPE CR-PCa material to identify potential therapeutic targets. Validation studies would further shed light into the biological understanding of the disease and its plausible treatment options.


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Background: The discovery of pivotal genetic alterations and the understanding of their potential role in cancer is leading to remarkable successes in translational medicine.

Materials and Methods: We initially evaluated the efficacy of a panel of novel TaqMan® Mutation Detection assays (TMDA) based on competitive allele specific TaqMan PCR to detect KRAS mutations. 96 archival FFPE specimens were examined and analysed for presence of KRAS mutations using (a) pyrosequencing, (b) custom conventional TMDA assays and (c) TMDA. Overall there was >96% concordance across the methodologies used.

Next we used TMDA assays in conjunction with the OpenArray system to rigorously detect the 3 most common KRAS mutations – G13D, G12D and G12V.

We established proof of principle in a cell line model by performing serial dilutions of each mutated DNA in a background of wild type DNA (Nthy-ori). We followed this with TMDA assay and OpenArray analysis in n = 25 clinical formalin fixed paraffin-embedded (FFPE) samples.

Results: We have shown that TMDA assays can be used in conjunction with OpenArray for low copy mutation detection. A clear distinction can be made between the differentially mutated sample cohorts with minimal cross talk between assays.

Conclusions: This combined approach represents a feasible option for diagnostic assessment of KRAS mutation in FFPE cell samples. The increased sensitivity and specificity can facilitate accurate mutation detection of heterogeneous CRC samples. Its sensitivity renders it a potential option for the future detection of circulating tumour cells or tumoural DNA in peripheral blood samples.


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Background: High grade undifferentiated pleomorphic sarcomas (HGUPS) represent a recently renamed subgroup that accounts for approximately 5% of adult soft tissue sarcomas. Apart from its known cytogenetic complexity, there is still a lack of in the understanding of its molecular biology. PI3K-AKT and MAPK growth-factor signalling are considered two major mechanisms of proliferation in sarcomas. Disturbances in cell cycle genes may also play a crucial role. New biomarkers in such group of tumors are an unmet medical need.

Methods: We retrospectively analyzed clinical data from 42 patients with surgically resected stage II and III HGUPS. Immunohistochemical staining in formalin-fixed and paraffin-embedded tissue was done in all cases for 4EBP1, p4EBP1, S6K, 4E-BP4, 4E-BP4, eIF4E, eIF4E, p16INK4a and ki-67. The levels of expression were evaluated as percentage and intensity of stained cells (Hscore). p16INK4a and ki-67 were evaluated as a percentage due to the homogeneity in the staining.

Results: Patients characteristics were: median age 72 (32–96); gender: 20 male, 15 female and 2 unknown; median tumor size 9 cm (2–30); Grade 2, 6 pts (16%), Grade 3, 27 pts (73%), unknown 4 pts (10%); stage IA 11 pts (30%), stage IB 3 pts (8%), stage II 22 pts (59%); 21 pts received adjuvant radiotherapy (57%) and 13 adjuvant chemotherapy (35%). In the univariate analysis: low pERK (Hscore < 40%) and ki-67 (>40%) expression were also associated with overall survival (OS) (p = 0.012, p = 0.001 and p = 0.002). Furthermore, low pERK expression was associated with early relapse at 1 and 3 years (p = 0.047 and p = 0.23). In the multivariate analysis: p4EBP1, eIF4E and p4EBP1 were independent prognostic factors of DFS (p = 0.001, p = 0.001 and p = 0.004) and OS (p = 0.011, p = 0.002 and p = 0.006). High K667 and p16INK4a levels were associated only with less OS (p = 0.001 and p = 0.001).

Conclusions: pERK, p4EBP1, eIF4E, p16INK4a and K67 are independent prognostic factors of HGUPS. We have found a strong correlation of all these proteins with DFS and OS. Signalling through MAPK and p4EBP1 pathway might therefore be a critical targetable option in HGUPS patients.
FGFR1 Amplification in Metastatic Squamous Cell Carcinoma of the Head and Neck – a Potential Target for a Rational Therapy? 

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Background: Currently, patients with FGFR1 amplified squamous cell lung cancers (SCLC) are treated in phase I/II clinical trials using small molecule inhibitors. Of interest, SCC of the larynx share common molecular alterations with squamous cell head and neck cancers (HNSCC). Aim of our study is to assess if HN-SCC also harbor FGFR1 amplifications. Furthermore, we identify a HN-SCC cell line harbouring FGFR1 amplification and inhibit cell proliferation using a small molecule inhibitor.

Methods: We put together a cohort of 227 patients suffering from HN-SCC, with 40 of them suffering from metastatic disease. Primary tumors and, where available, metastatic tumors were assessed for FGFR1 copy number status using fluorescence in-situ hybridization (FISH). We tested different cell lines for FGFR1 amplification status and inhibited these with small molecule inhibitors.

Results: 20.3% of primary HN-SCC displayed FGFR1 amplifications. Of interest, almost all metastases revealed a FGFR1 amplification if the corresponding primary tumor harbored the amplification. The cell lines HN and SCC showed by technical and independent FGFR1 amplifications and cell proliferation and migration was inhibited with small molecule inhibitors.

Conclusion: FGFR1 amplification frequently occurs in primary and metastatic HN-SCC and proves as a potential target for small molecule therapy in nonresectable disease. Furthermore, cell growth and migration of FGFR1 amplified cell lines is inhibited with small molecule inhibitors. Further functional studies and subsequent clinical trials are needed for further validation of our findings.

MicroRNAs Profiling in the Screening of Potential Biomarkers for PCa Diagnosis and Prognosis


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Background: MicroRNAs (miRNAs) are non-coding, single-stranded RNAs that negatively regulate gene expression at the post-transcriptional level by miRNAs that could be used as potential biomarkers for PCa diagnosis and prognosis.

Material and Methods: Total RNA was obtained from 10 normal prostates (NP; ⩽ 50 years) and 50 PCa (65±17 years old) samples and analyzed using Affymetrix's GeneChip®miRNA 2.0 Array. The expression of those miRNAs differentially expressed was validated by qRT-PCR.

Results: Differentially expressed miRNAs were involved in the tumorigenesis of hereditary diffuse gastric cancer (HDGC) and were considered as potential biomarkers for PCa diagnosis and prognosis. miR-182 and miR-187 showed coherent levels in cancer and normal tissue. List of miRNAs found to be overexpression in PCa included miR-21, miR-10b, miR-125a, miR-125b, miR-146a, miR-195, miR-200a, miR-200c, and miR-200d.

Conclusions: These data indicate that these miRNAs are upregulated in PCa and might be useful for PCa diagnosis.

Development of a Quantitative RON SRM Assay for Use in Formalin Fixed Tumor Tissues


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Introduction: RON and Met are implicated as mediators of tumor progression/metastasis in cancer. Over-expression of each is prognostic of poor survival in resected/metastatic cancers. Expression of each in preclinical models/early phase trials predicts response to RON/Met specific inhibitors. To date, clinical quantification of protein in formalin fixed paraffin embedded (FFPE) tissues is limited to immunohistochemistry (IHC), and is semi-quantitative at best. IHC of multiple proteins is laborious, time consuming, wasteful of scarce tissue, and costly. Other protein quantification methods (ELISA, ECL) would require standard tissue processing for analysis. We sought to develop a quantitative mass spectrometric (MS) assay for RON utilizing Liquid Tissue – Selected Reaction Monitoring (SRM), with subsequent multiplex quantification of RON, Met, and other previously validated proteins in a panel of gastroesophageal cancer (GEC) cell lines and tissues.

Material and Method: Using trypsin digestion/mapping of recombinant RON, we identified unique peptide sequences, and built quantitative MS assays which could be multiplexed into a single SRM analysis of 1 ug of tumor protein. Assays were preclinically validated on 10 different formalin fixed (FF) cell lines. The final assay was validated and the N-terminal RON SRM demonstrated an LOD/LOQ of 62/125.

Then we tested the RON assay using a panel of FF GEC cell lines previously characterized by immunoblot (IB) and IHC FFPE pellet. In addition to RON, we multiplexed SRM quantification of Met, EGFRL, HER2, HER3, IGF1R, and cSRC. We evaluated 15 GEC lines including three AGS lines: wild type (AGS-WT), scrambled shRNA (AGS-SC) and RON shRNA knockdown (AGS-KD) to assess ‘post-treatment’ changes in oncogene expression profiles. We then evaluated 20 GEC human cancer tissues and 5 paraneoplastic normal tissues using laser capture microdissection of the target material from a single unstrained 10 μm thick section per sample.

Results and Discussion: In the initial analysis, 4/10 cell lines (HC8827, Colo205, HT29, A431) expressed N-terminal RON (~250 amol/μg cell protein). Validation of the RON SRM assay on GEC cell lines revealed very high concordance when compared to IB/IHC measurement. The AGS-WT/SC cells showed comparable levels of N-terminal RON (84/82 amol/μg cell protein), while RON was not detected in AGS-KD cells. Correlation of IB with RON MS assay data will be presented. Measurement of RON in the GEC tissues correlated well with IHC. Multiplexed oncogene quantification of all cell lines and tissues, along with expression profile changes in the AGS-KD line compared to AGS-WT/SC will be presented.

Conclusion: These data demonstrate a sensitive, accurate, and quantitative assay to measure RON in FFPE tissues. Multiplexed oncogene quantification of these tumors was feasible and expedient using limited tissue, and is a novel clinically applicable approach for tumor characterization for baseline and post-treatment assessment.

Analysis of Germine E-cadherin Mutations in Brazilian Patients With Hereditary Diffuse Gastric Cancer

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Introduction: E-cadherin mediates cell–cell adhesion by being an essential component of the intercellular adhesion complexes. Germline mutations of the E-cadherin gene (CDH1) are involved in the tumorigenesis of hereditary diffuse gastric cancer (HDGC). HDGC is an autosomal dominant disorder that accounts for 1% of all cases of gastric cancer (GC). Although uncommon, this syndrome has high penetrance, poor prognosis (5-year survival rate of 10%), early age at presentation and ineffective screening tools. Therefore, the only alternative to change the natural history of this syndrome is the identification of genetic markers to allow early detection of germline mutations in the HDGC. The objective of this study was to characterize the profile of E-cadherin germline variants in patients with clinical criteria for HDGC attended at the National Cancer Institute (INCA) of Brazil.

Materials and Methods: Seventeen patients fulfilling clinical criteria for HDGC (according to the International Gastric Cancer Linkage Consortium and the expanded Brooks-Wilson criteria) were retrospectively identified from among gastric cancer patients seen at the Instituto de Salud Carlos III; ACCOMP12/029, Generalitat Valenciana; and Astra Zeneca, Spain.

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including all intron/exon junctions was amplified using PCR and screened for mutations by direct sequencing.

Results and Discussion: CDH1 germline variants (point mutations and deletions) were identified in six of 17 patients: a case of missense mutation in exon 8 (1037A>C), two cases of missense mutation in intron 1 (48 +5 C>G and a deletion in intron 5 (del 48 +63−5). In addition, six polymorphisms, previously published, were identified in nine patients (48 +6 T>C, 531+10 G>C, 943C>G, 1849G>A, 1896C>T, 2253C>T). The low rate of mutations detected in our patients group (with conclusive clinical criteria for HDGC) suggests that other changes in CDH1, as large deletions, may be involved in these probands.

Conclusion: The identification of genes predisposing to familial cancer is an essential step towards understanding the molecular events underlying tumorigenesis and is critical for the clinical management of affected families. Our results emphasize the need to incorporate other methodologies to characterize the variation profile in CDH1 in HDGC patients.

[822] ETV5 and RUNX1 Expression as Potential Biomarkers Associated With Myometrial Invasion in Type I Endometrial Carcinoma


Introduction: Endometrioid adenocarcinoma is the most common type of cancer in the endometrium, accounting for 80 to 90% of cases. Most of these tumors are diagnosed in early stages and have a favorable prognosis, but some may present an unexpected recurrence, with resistance to treatment. Prognosis is based solely on clinicopathological features, such as myometrial invasion. ETV5/ERM and RUNX1/AML1 expression and myometrial invasion.

Material and Methods: We identified 169 patients with endometrioid adenocarcinoma who were attended at the Brazilian National Cancer Institute from 2007 to 2009. ETV5/ERM and RUNX1/AML1 protein expression and myometrial invasion.

Results and Discussion: ETV5 expression was detected in 98% of tumors (100%) whereas RUNX1 was expressed in normal (58.3%) and tumor tissue (100%). ETV5 presented both nuclear and cytoplasmic expression, whereas RUNX1 presented only nuclear expression (although in normal tissue it was expressed in cytoplasm). However, there was no difference in ETV5 or RUNX1 expression between stages IA and IB tumors. ETV5 expression was higher in 3+ IHC positive tumors, whereas RUNX1 expression was not correlated with IHC expression.

Conclusion: There is no association between ETV5 and RUNX1 expression and myometrial invasion in early stages of endometrioid adenocarcinomas.

[823] Study of the Response of Melanoma Lines to BRAF Inhibitors

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Introduction: The serine-threonine protein kinase, BRAF, is a frequently mutated oncoprotein, found in cancers of the skin, thyroid and large intestine. It is especially prevalent in malignant melanoma, where it occurs in 40–60% of the cases. The most common genetic alteration is a valine to glutamate substitution at position 600 of the kinase (V600E). This substitution, seen in ninety percent of the BRAF cases, leads to constitutive activation of the kinase, and the downstream MEK-ERK pathway. Appropriately, several drugs have been developed to inhibit BRAF with a broad range of outcomes in clinical trials. Our aim is to better understand, and ultimately predict, the response of patients to BRAF targeted therapy.

Materials and Method: We used a panel of 44 melanoma lines, comprising of 28 BRAF mutants, 12 NRAS mutants and 4 lines that were wild type for these two genes. Besides BRAF and NRAS, we have the mutational status of 85 other commonly mutated cancer genes for these lines. We also associated a set of RAF inhibitors, including PLX4720 and PLX4032, which preferentially target the V600E mutant. The melanoma lines were treated with the RAF inhibitors for 72 hrs over a nine point, 256-fold concentration range. We calculated the half maximal inhibitory concentrations (IC50s) for each cell-line-drug interaction. Caspase galo 3/7 (Promega) was used to compare levels of apoptosis between the cell lines.

Results and Discussion: The IC50s for the BRAF mutant lines varied from the low nanomolar to the micromolar range. This is also reflected in data from clinical trials, where 81% of V600E-bearing melanoma patients showed partial or complete tumor regression, but some patients did not respond. We are currently looking at the MEK-ERK signaling pathway to tease out differences between the sensitive and resistant groups of lines. The caspase 3/7 data revealed, that although many lines were growth inhibited by PLX4720, only a handful of them underwent apoptosis. Some cell lines were very sensitive to certain RAF inhibitors, but resistant to others in our panel, revealing differences in the activities of RAF inhibitors.

Conclusion: Our screen of 44 melanoma cell lines and 9 RAF inhibitors is a very comprehensive study that allows us to compare and contrast responses of cell lines and link them to their genotype and signaling profiles.

[824] Cancer Cell’s Surface Traits – an Ally in Designing Targeted Cancer Therapies – a GnRH Based Approach


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Introduction: The gonadotropin releasing hormone receptor (GnRHR) has been shown to be overexpressed in various cancer types, providing opportunities for targeted therapy. The synthesis of conjugate molecules containing gencmicbatin in proof-of-concept studies including NMR mapping, in vitro and in vivo stability, biodistribution and efficacy.

Materials and Methods: Given that gencmicbatin containing gencmicbatin were synthesized and evaluated by NMR in order to assess in the resulting analogues potential conservation of microenvironments responsible for binding to the GnRHR. The efficacy of the GnRHR conjugates in vitro was based on the MT assay using three prostate cell lines: WPE−1-NB26−3 (stably expressing the GnRHR), DU145 and PC3. The GnRHR-gencmicbatin conjugate and its analogues were incubated in mouse plasma in order to examine the release of gencmicbatin. For the examination of cellular uptake rate of the GnRHR conjugates and pharmacokinetic evaluation, LC-MS/MS methodologies were established. Regarding the in vivo pharmacokinetic evaluation, mice were dosed with GnRHR-gencmicbatin or gencmicbatin (10 mg/kg) and blood samples were analyzed by LC-MS/MS. For the in vivo efficacy studies, SCID mice were injected with WPE−1-NB26−3 or DU145 cells to generate tumours. Pharmacological treatment was subsequently initiated and efficacy was evaluated based on tumour volumes.

Results and Discussion: The preferential antiproliferative effect of GnRHR agonists in cells over-expressing the GnRHR and the efficacy of a GnRHR-gencmicbatin conjugate were demonstrated. The effective release of gencmicbatin from the GnRHR-gencmicbatin conjugate was shown in plasma incubation studies, a process that can be adjusted by synthetically altering the linkers employed in the conjugated analogues. Gencmicbatin derived from the conjugate product illustrated a slower cell uptake rate compared to gencmicbatin alone in the WPE−1-NB26−3 cells suggesting a potential benefit (efficacy vs toxicity).

Gencmicbatin from GnRHR-gencmicbatin conjugate appears to have a slower release in blood after in vivo administration in mice compared to gencmicbatin alone which could potentially lead to enhanced efficacy. Efficacy of gencmicbatin in xenografts from WPE−1-NB26−3 cells has been demonstrated and follow up studies with the conjugates are pending.

Conclusion: The main goal of this project is the development of a targeted therapy for prostate cancer. To this end, we achieved the synthesis of efficacious conjugate molecules and proceeded with proof-of-concept experiments that allow us to expand the work to other types of cancers and other types of receptors.
Exome Sequencing of T-LGL Leukemia Patient Revealed ANGPT2 as a Possible Mutational Target

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Introduction: T-cell large granular lymphocyte (T-LGL) leukemia is a rare, clonal disease characterized by the expansion of mature CD3+CD8+ cytotoxic T-cells. It is often associated with autoimmune disorders and immune-mediated cytopenias. Our recent findings suggest that up to 40% of T-LGL leukemia patients harbor mutations in the STAT3 gene. In this project, we aimed to screen all patients for STAT3 mutationnegative T-LGL leukemia patient by whole exome sequencing.

Material and Methods: The index patient is a 40 year-old male with untreated T-LGL leukemia. At the time of diagnosis he had absolute lymphocytosis (8.9 x 10^9/l) and a polyclonal B-cell expansion. Exome sequencing was performed with the Illumina HiSeq2000 sequencing platform. Candidate somatic mutations were identified with a bioinformatics pipeline consisting of BWA for sequence alignment, Samtools for alignment filtering and Varscan for somatic mutation calling. Samples from 9 additional T-LGL leukemia patients were used for further screening of confirmed somatic mutations.

Results: The exome sequencing revealed 17 nonsynonymous nucleotide variants from which 5 variants were chosen for validation based on p-value (p<0.0001) and medical relevance. Somatic heterozygous mutations in ANGPT2, ACCN5, NELL1 and SPG20 genes were confirmed by capillary sequencing. None of the mutations were found in other T-LGL leukemia patients (n=9). The missense mutation in ANGPT2, presenting with the lowest somatic p-value (1.06E-07) and highest variant frequency (34%), was the most relevant candidate involved in the pathogenesis of leukemia. It causes the formation of a variant protein as a positively charged lysine is changed into a negatively charged glutamic acid residue (p.K436E). The mutation occurs on the surface of ANGPT2 within the well-conserved fibrinogen C-terminal domain. This domain binds the receptor TIE2 and the change in the polarity induced by p.K436E mutation is likely to affect the binding of TIE2 by ANGPT2.

Conclusions: The fibrinogen C-terminal domain of ANGPT2 is a putative mutational target in T-LGL leukemia. Screening of a larger T-LGL leukemia patient cohort (n=100) is ongoing to confirm the impact and recurrence of ANGPT2 mutations in T-LGL leukemia.

RAD51 G172T Polymorphism – a Prognostic Value in Cervical Cancer

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Background: Cervical cancer is one of the most common cancers diagnosed in women worldwide. Mammalian cells are constantly exposed to a wide variety of genotoxic agents from both endogenous and exogenous sources. The RAD51 protein is required for meiotic and mitotic recombination and plays a central role in homology-dependent recombinational repair of double-strand breaks (DSBs). Given the functional relevance of the DNA repair system on carcinogenesis, several associations between genetic polymorphisms of DNA repair genes, cancer risk and response to therapy have been intensively evaluated. This is the first study evaluating the role of the RAD51 G172T genetic variants in cervical cancer prognosis and clinical outcome of cervical cancer patients.

Material and Methods: We analysed RAD51 G172T polymorphism genotypes in cervical cancer patients who underwent a platinum-based chemotherapy in combination with radiotherapy. Genotyping was performed by TaqMan® Allelic Discrimination methodology.

Results: Concerning the overall survival rates found using Kaplan–Meier method and Log Rank Test, we observed that the mean survival rates were statistically different according to the patients RAD51 genotypes. The group of patients carrying the T allele present a higher mean survival rate than the other patients (102.3 months vs. 86.4 months, P = 0.002). Using the Cox regression analysis, we found an increased overall survival time for T-carrier patients, when compared with GG genotype, with tumor stage, age and presence of lymph nodes as covariates [hazard ratio (HR), 0.373; 95% CI, 0.181–0.770; P = 0.008]. Among patients (n=193), RAD51 G172T genotype frequency distributions were not under the influence of clinicopathologic characteristics, namely, treatment response (P = 0.508), recurrence (P = 0.150) and tumor stage (P = 0.250).

Conclusions: This is the first study evaluating the role of the RAD51 G172T genetic variants in cancer prognosis and clinical outcome of cervical cancer patients. Our results indicate an influence of the RAD51 genetic variants in overall survival of cervical cancer. Thereby, RAD51 G172T genotypes may provide additional prognostic information in cervical cancer patients who underwent cisplatin-based chemotherapy in combination with radiotherapy.

KIT Mutation in a GIST From A Patient With SDHD-associated Carney-Stratakis Syndrome

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Background: Gastrointestinal stromal tumors (GIST) are mesenchymal neoplasms of the gastrointestinal tract characterized by the oncogenic activation of KIT or PDGFRA. While the majority of GIST are sporadic, some are inherited, either alone (familial GIST, with germline KIT/PDGFRA mutation) or as component of syndromes, such as the Neurofibromatosis-1 and the Carney-Stratakis Syndrome (CSS). CSS is a rare autosomal-dominant condition characterized by paraganglioma and GIST CSS has been associated with germline mutations of the succinate dehydrogenase complex subunits genes (SDHB, SDHC and SDHD). Notably, different from sporadic GISTs, the css-associated GISTs have been reported to lack KIT/PDGFRa gene mutations.

Materials and Methods: A young (38 years) female patient with GIST and multiple paragangliomas was analyzed for SDH. KIT and PDGFRA gene mutations. Pathological, genetic and clinical follow-up was performed.

Results: The patients carried a germline mutation (p.Cys150TyrfsX42) of SDHD. This mutation resulted in the loss of mitochondrial complex II, as indicated by the negative SDHB immunostaining in GIST and paragangliomas. Analysis of GIST lesions revealed that concurrent to the SDHD mutation, the tumor carried a somatic mutation of KIT exon 11 (p.Trp557_Val559delinsPhe). Imatinib treatment resulted in a significant response.

Conclusions: Here we report a case of CSS syndrome in which the GIST lesion carried a canonical KIT mutation. Different from most syndromic GIST, this patient responded well to Imatinib treatment. Thus, different from what reported so far, this case indicates that the presence of a KIT mutation in a GIST patient should not a priori rule out the possibility of Carney-Stratakis syndrome in suggestive clinical contexts. Of course, this impacts on the surveillance protocol for both the patient and the relatives. By converse, the condition of CSS does not preclude the possibility that the tumor may be responsive to Imatinib treatment because of the possible somatic gain of KIT/PDGFRA sensitizing mutations.

Involvement of Endoplasmic Reticulum in the Antitumor Activity of the Alkylysophospholipid Edelfosine against Ewing Sarcoma

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Introduction: Ewing sarcoma (ES) is the second most common type of bone cancer in children and adolescents. Whereas cure rates for patients with localized tumors are around 70%, survival rates for patients with metastasis or relapse are poor despite intensive chemo- and radiation therapy, thus demonstrating a clear need for new therapies. Edelfosine (1-O-octadecyl-2- O-methyl-rac-glycer-3-phosphocholine) is the prototype of a promising family of synthetic antitumor compounds, collectively known as alkyl-lysophospholipid analogs (ALPs), which act at the cell membrane and induce apoptosis.

Materials and Methods: Apoptosis was assessed by the percentage of hypodiploid cells by flow cytometry, and biochemically characterized by Western blot and solid phase c-Jun amino terminal kinase (JNK) assay. Subcellular localization of edelfosine was determined by confocal microscopy using a fluorescent analog and transfecting with a RFP-coupled endoplasmic reticulum marker. ES xenograft models in SCID mice were assayed following oral treatment with edelfosine for 21 days at different doses (30 and 40mg/kg/day).

Results and Discussion: We have found that ALPs induced apoptosis in ES cell lines, ranking edelfosine > perifosine > erucylphosphocholine > miltefosine for their proapoptotic activity. Edelfosine induced a potent caspase-dependent apoptotic response in ES cells, whereas and structurally-related inactive edelfosine analog fail to promote cell death. Edelfosine accumulated in endoplasmic reticulum and triggered an endoplasmic reticulum stress response, leading to caspase-4 and c-Jun activation, Bax and Bid induction, growth arrest and DNA damage-inducible gene 153 (GADD153) up-regulation, and eventually to apoptosis. Sustained JNK activation was detected following edelfosine treatment, and inhibition of JNK diminished edelfosine-induced cell death.

apoptosis. Edofosine treatment led to the generation of the p20 caspase-8 cleavage fragment of BAF31, thus linking prosaposin signals between ER and mitochondria. Edofosine treatment also led to cytochrome c release, caspase-9 activation and generation of reactive oxygen species, suggesting the involvement of mitochondria in the apoptotic process. Furthermore, oral administration of edofosine showed in vivo antitumor activity in an Es xenograft animal model.

Conclusion: These results indicate a significant anti-apoptotic activity of edofosine against ES that is mediated by caspase activation and endoplasmic reticulum stress.

**Polo-like Kinase 1 – a Potential Therapeutic Target for the Management of Patients With Triple Negative Breast Cancer**

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**Background:** Breast cancers are composed of molecularly distinct subtypes with different clinical outcomes and responses to therapy. Our aim is to discover potential therapeutic targets for triple negative breast cancer (TNBC), a poor prognosis subgroup with no targeted therapy available yet.

**Material, Methods and Results:** Gene expression profiling on a cohort of 160 breast cancer subtypes including 40 TNBC revealed that the polo-like kinase 1 (PLK1), a protein kinase, is specifically over-expressed in TNBC compared to the other breast cancer subtypes. High PLK1 expression was confirmed at a proteomic level, with a more than 2 fold increase in protein amount and tissue volume. In TNBC cell lines, PLK1 is over-expressed as monolayers, RNA-mediated PLK1 depletion or small compound (BI-2536)-mediated PLK1 inhibition induced a decrease in phosphorylated H2AX, G2/M arrest and apoptosis, resulting in a decrease in cell viability. In addition, at the medium through colony assay showed that PLK1 silencing impaired tumorigenicity of TNBC cell lines. With cells grown in extracellular matrix gels (Matrigel), BI-2536 induced apoptosis specifically in TNBC cancerous versus normal cell lines. The in vivo anti-tumor effect of BI-2536 was investigated in an orthotopic xenograft model derived from patient’s biopsies. When administrated as a single agent, the PLK1 inhibitor significantly impaired the tumor growth. Most importantly, administration of BI-2536 in combination with adriamycin + cyclophosphamide chemotherapy led to 100% complete response (9/9 mice).

**Conclusions:** Altogether, our observations point out that PLK1 may represent an attractive therapeutic target, in association with conventional chemotherapy, for the management of patients with TNBC.

**Bi-phasic Profile of MnSOD During Tumor Progression in Prostate Cancer**

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**Background:** Starting therapeutic approach in prostate cancer includes anti-androgenic treatments leading to an initial tumor regression. However after tumor relapses and becomes androgen-insensitive with an increase in a cell population with neuroendocrine differentiation (NED) features. Reactive oxygen/nitrogen species have a role in cell signaling and in decision of cell fate (survival/proliferation/differentiation) and their levels are controlled by antioxidant enzymes including superoxide dismutases (cysotolic CuZnSOD and mitochondrial MnSOD) which are particularly important. Previously, our group has described that MnSOD is instrumental for NED, but its exact role in tumor progression is not clear.

**Objectives:** To determine the role of MnSOD during the tumor process in patient samples with different Gleason grades of prostate adenocarcinoma and its correlation with AR and PSA levels.

**Methods:** Frozen prostate tissue samples (n = 107) from prostate biopsy were acquired from the Tumor Bank of Central Hospital of Asturias (HUCA, Oviedo, Spain). The distribution of samples was as following: normal (22), hyperplasia (28), Gleason grade lower than 6 (62), Gleason 6 (20), Gleason 7 (19) and Gleason grade over 7 (15).

**Results:** PSA levels throughout the tumor progression (Gleason grade) do not show a constant profile. CuZnSOD show a decrease with Gleason score while MnSOD protein levels show an initial drop in hyperplastic samples and then increase dramatically at both RNA and protein levels. Interestingly this increase is almost inversely to that of catalase, while GPx also drops throughout the tumor progression. Finally AR protein levels increase initially but then it is re-stored to normal levels. Only HIF-1α shows a correlation with Gleason score.

**Conclusion:** Our results point out that MnSOD does not operate as a tumor suppressor gene but rather on the contrary as its protein and mRNA levels are increased at the medium high tumour stage, showing a bell-shape and not a linear correlation with Gleason grade. Balance between MnSOD and catalase appear crucial for prostate tumor growth, thus confirming that O2−/H2O2 ratio as a critical regulator of cell proliferation and metastasis.

**Telomerase Activity Has Prognostic Quality for Glioblastoma Patients Preferentially of Younger Age**

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**Introduction:** Glioblastomas (GBM) are the most frequent and unfortunately most aggressive primary brain tumors. Although morphologically widely identical at the cellular level, glioblastomas show heterogeneity in their molecular pattern. Clinically, this is reflected by individually different courses of disease accompanied by survival times varying between a few weeks and up to 10 years. In search of prognostic biomarkers we focussed on telomerase activity (TA) and the catalytic subunit human telomerase reverse transcriptase (hTERT). The enzyme telomerase, reactivated in most cancer cells maintains telomere length by adding a specific sequence of six nucleotides to the ends of chromosomes thus promoting cell immortalization and oncogenesis.

**Material and Methods:** In the present study we investigated GBM tumor tissue from patients (n = 100) operated at our institution. Telomerase activity (TA) and hTERT expression and length of telomeres (TL) were analyzed using the Telomeric Repeat Amplification Protocol (TRAP), RT-PCR and quantitative PCR, respectively. Additionally these parameters were correlated with disease progression of glioblastoma patients.

**Results:** Sixty-one percent of GBMs were positive for both hTERT mRNA expression and telomerase activity. Despite of inconsistency in 2 cases (negative hTERT expression but detectable TA) the correlation between hTERT and TA was highly significant (Fisher’s exact test, P < 0.0001). Measurement of telomere length revealed a statistically significant difference between the hTERT/TA positive and negative subgroups showing markedly longer telomeres in the hTERT/TA negative cohort (unpaired t test; p < 0.0001). Additionally, similar results were achieved by comparing age groups (<60 years) patients (unpaired t test; p < 0.0001). Moreover, analysis of Kaplan Meier survival estimates revealed a significant survival benefit for patients whose tumors lack either hTERT expression (p < 0.005) or TA (p < 0.0008) preferably in a subgroup of patients of younger age.

**Conclusion:** In summary, telomerase activity regulated by hTERT expression was observed in a subgroup of GBM patients. Moreover, these data suggest that GBMs showing reactivated telomerase provoke a more aggressive clinical type of tumor reflected by significantly shorter telomeres and associated with significantly shorter overall survival. Thus, hTERT and telomerase activity might have quality as prognostic biomarker especially in the subgroup of patients of younger age.

**Relationship Between Circulating and Tissue miRNAs in a Murine Model of Breast Cancer**

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**Background:** miRNAs are small non-coding RNAs that have been shown to be key regulators of tumorigenesis. They are aberrantly expressed in both the circulation and tissue of patients with cancer, however little is currently known about the relationship between circulating and tissue miRNAs. The aim of this study is to quantify circulating and tumour tissue miRNA expression in a murine model of breast cancer.

**Materials and Methods:** A murine model of hormone receptor negative breast cancer was established using MDA-MB 231 cells in female athymic nude mice (n = 20). Animals received either an injection of 2 × 10^8 MDA-MB 231 cells into the second thoracic mammary fat pad (n = 8, MFP) or a subcutaneous injection of 4 × 10^6 MDA-MB 231 cells into the right flank (n = 7, SC). A control group were not injected with tumour (n = 5). Tumour volume was measured weekly and blood sampling performed at week 1, 3 and 6 following tumour induction. Animals were sacrificed at week 6 and tumour tissue (n = 15), lungs (n = 15) and enlarged lymph nodes (n = 3) were harvested. MicroRNA was extracted from all blood and tissue samples (n = 85). For this purpose, an extraction protocol for miR-497, 195, 215, 195, and endogenous controls miR16 and let7A, was carried out and expression quantified using Q-PCR.

**Results:** miRNA expression was found to be significantly increased in tumours versus normal tissue (p < 0.05). MI-R-103 expression was significantly increased in SC tumours versus normal tissue (p < 0.05) and further increased upon disease metastasis with the highest levels detected in enlarged lymph nodes (p < 0.05). There was no significant change in circulating miR-221 in tumour bearing animals, with
Conclusions: This study highlights the importance of miRNAs in breast cancer. Circulating miR-497 with miR-195 was also observed (p < 0.01, r = 0.4). The aim of this study was to investigate the relevance of WRAP53 in breast cancer.

Material and Methods: Tissue microarray constructed from 167 primary breast tumors collected at Ullevål University Hospital was immunochemically (IHC) analyzed to assess the expression pattern of the WRAP53 protein. The results obtained were further validated by IHC in a larger series of 668 breast tumor samples (Oslo Micrometastasis Study) and by Real-time PCR in a subset of samples. Statistical analysis was performed using SPSS.

Results: The WRAP53 protein was found localized both in the nucleus and the cytoplasm of the breast carcinoma cells. Interestingly, breast cancer patients with positive nuclear staining of WRAP53 had a statistically significant better survival than patients with negative nuclear staining.

The effect of combining nuclear and cytoplasmic protein localization was even more pronounced. WRAP53 positive nucleus in combination with negative cytoplasm gave the best prognosis, and WRAP53 negative nucleus combined with positive cytoplasm gave the poorest prognosis. A striking observation was that nuclear WRAP53 was found to be a better prognostic marker in TP53 mutant vs. TP53 wild type patients, and in ER negative vs. ER positive patients. The nuclear localization of WRAP53 was further shown to be an independent marker of prognosis in multivariate analysis.

Conclusion: The cellular localization of the WRAP53 protein showed a significant impact on breast cancer survival, and WRAP53 may be used to predict outcome and potentially influence treatment in patients with TP53 mutation. Our findings elucidate the role of WRAP53 in cancer, and to further uncover the underlying biology, functional investigation is required.

Synergistic Response Induced by Quercetin and ABT-737 in Leukemic Cell Lines and in B-Cells Isolated From Chronic Lymphocytic Leukemia

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Background: Chronic Lymphocytic Leukemia (CLL) is the most frequent form of leukemia in adult population and despite numerous studies it is considered an incurable disease. Since CLL is characterized by overexpression of pro-survival Bcl-2 family members, treatment with their antagonists, such as ABT-737, may represent a promising new therapeutic strategy. ABT-737 binds and inhibits with high affinity Bcl-2, Bcl-XL, and Bcl-W, but with lower affinity Mcl-1 and Bfl-1. Previous studies from our group demonstrated that quercetin, a flavonoid naturally present in food and beverages belonging to the large class of phytochemicals, was able to sensitize B-cells isolated from CLL patients to apoptosis when associated with death ligands or fludarabine, through a mechanism involving Mcl-1 down-regulation. Therefore, we hypothesized that quercetin in combination with ABT-737 could enhance apoptosis in leukemic cell lines and B-CLL cells.

Material and Methods: Leukemic cell lines (HPB-ALL, Jurkat, U-937, K562, HL-60) and B-cells isolated from 10 CLL patients were treated with quercetin (10-25 mM), ABT-737 (1-1000 nM) and their association. Cell viability and apoptosis were measured by neutral red assay, Annexin V positivity, activity of caspases 9 and 3. Combination index (C.I.) was calculated to assess synergistic effect of the two molecules. Gene and protein expression were measured by reverse transcriptase polymerase chain reaction and immunoblotting, respectively.

Results: The association between quercetin and ABT-737 synergistically enhanced apoptosis in all leukemic cell lines investigated and B-CLL cells (C.I. ≤ 0.1). At the same concentrations, the combined treatment did not induced any cytotoxic effect on peripheral lymphocytes from healthy donors. We selected HPB-ALL cells, which showed high sensitivity to the co-treatment to investigate the molecular mechanism triggered by quercetin. The molecule down-regulated Mcl-1 mRNA and protein expression) through the inhibition of PI3K/Akt and/or MEK/MAPK signaling pathways, leading to Mcl-1 instability. The same mechanism was confirmed in B-cells isolated from CLL patients.

Conclusion: The effect of quercetin to synergistically enhance apoptosis in association with ABT-737 can be partially attributed to the ability of the molecule to inhibit PI3K/Akt and/or MEK/MAPK signaling pathways leading to Mcl-1 stability. These results may open new applicable clinical perspectives in CLL therapy.

Improving the Reliability of Gene Expression Profiles From FFPE Samples Using Alternative Chip Description Files

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Background: Formalin fixed paraffin-embedded (FFPE) tissue specimens are collected for routine diagnostic histopathology and represent a unique source of material for molecular biomarkers development but, as a consequence of fixation, RNA quality can be low, making a genome-wide expression analysis technically challenging. Recently, biologically meaningful gene expression data from FFPE clinical samples were obtained using the Affymetrix HG-U133 Plus 2.0 microarray platform after amplification with the Nugen WT-Ovation FFPE System, nevertheless, high discrepancies remain between frozen and FFPE expression data. We hypothesized that an optimized processing using alternative Chip Description files (CDFs) could improve the quality of downstream analysis.

Material and Methods: Using the affx/eqc/bioconductor package, we created two alternative CDFs, the first using all probes uniquely mapping to RefSeq transcripts (RefSeq_all) and the second selecting, among these, the five probes closest to 3′-end and in any case mapping within 300 bp from 3′-end (RefSeq_dist). As processing methods we considered MAS5, RMA and RMA as implemented in the affx and frma Bioconductor package, respectively. We tested our approach on a dataset of 56 matched frozen and FFPE DLBCL samples (GSE19246). For each combination of processing algorithm (MAS5, RMA, RMA) and CDF (standard, RefSeq_all and RefSeq_dist), we measured: (a) frozen-FFPE sample and frozen-FFPE probeset correlation; (b) frozen-FFPE fold change correlation and slope; (c) percentage of differentially expressed (DE) genes in frozen data called as DE also in FFPE data (P < 1e-4); (d) number of common genes among top-100 DE genes in frozen and FFPE data.

Results and Discussion: Globally, RMA and RMA clearly outperformed the MAS5 method. Moreover, better frozen-FFPE comparability was found using the RefSeq_dist alternative CDF instead of the standard CDF, to some extent due to the on average higher number of probes used to estimate the expression of each gene. Some parameters further improved when using the RefSeq_dist CDF but, looking at the overlap between genes identified as DE, no advantage was found.

Conclusions: We have demonstrated that the choice of the processing method and the use of alternative CDFs can improve the reliability of expression data from FFPE samples.

Correlation of Specific miRNAs Expression With Survival and Drug Resistance Related Protein Expression in Non-small Cell Lung Cancer

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Introduction: Lung cancer is the leading cause of cancer deaths worldwide. There are many suggested biomarkers for lung cancer examination but new targeted therapeutic approaches still require discovering new ones. Small non-coding miRNAs regulating protein levels in cells through induction of translational repression and miRNA degradation appear to be a useful target.
Material and Methods: Based on literature data and miRNA database, we have focused on analysis of 16 miRNAs (miR-21, miR-126, miR-205, miR-210, miR-215, miR-23b, miR-335*, miR-365-3p, miR-363, miR-451-3p, miR-548*, miR-548p, miR-576-5p, miR-590-5p, miR-655, miR-656, miR-944) that target genes involved in cell proliferation, differentiation, apoptosis and resistance. Our patient cohort consisted of 52 formalin-fixed paraffin-embedded (FFPE) samples from operable non-small cell lung cancer (NSCLC) patients treated in the University Hospital in Olomouc in years 1996–2000. Total RNA was isolated and after reverse transcription with miRNA-specific looped primers and preamplification PCR, real-time PCR was used for miRNAs quantification. Statistical methods were used to correlate the expression levels of miRNAs and different variables. For survival analysis the Kaplan–Meier method was used.

Results: The results of our pilot study suggested that expression of miR-205 positively correlated with overall survival of NSCLC patients (p < 0.059) in stage II patients and higher level of miR-944 expression (p < 0.017). The quantification of miR-205, miR-590-5p and miR-944 levels seems to be the useful diagnostic tool for better stratification of NSCLC patients and they might be used as diagnostic and prognostic markers.

Conclusions: This work was supported by grants GACR 303/08/H048, IGA MZ CR 10259-3 and CZ.1.05/2.1.00/03.0030.

[337] Notch Signaling and Chemoresistance in Gastric Cancer

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Introduction: In a recent study analyzing the prognostic significance of the expression of cancer stem cell (CSC) related genes in residual gastric tumor cells after neoadjuvant chemotherapy, Wnt and Notch signaling genes, among others, showed a prominent association with survival. The aim of this study was to assess selected genes for differential expression between pre- and post-chemotherapy gastric tumor tissues. In vitro we investigated the impact of Notch activity on chemosensitivity in gastric cancer cell lines.

Material and Methods: Expression of 9 genes was compared between corresponding pre- and post-therapeutic tumors from patients treated with neoadjuvant chemotherapy (CTx) demonstrating partial (n = 22) or minimal/no tumor regression (n = 22). miRNA was isolated from macrodissected FFPE tissues and gene expression was quantified by real time PCR using TaqMan® low density arrays. Immunohistochemical staining (IHC) for Notch2 was performed on corresponding pre- and post-CTx tumors from patients with sub-total, partial or minimal/no tumor regression (n = 22, each) and from patients not treated by CTx (n = 16) and evaluated by a semi-quantitative scoring system. Cytosensitivity of three gastric cancer cell lines to the γ-secretase inhibitor DAPT alone or in combination with cisplatin was determined by XTT or colony formation assays.

Results and Discussion: Differential expression analysis revealed an increase of NOTCH2 and POU5F1 from pre- to post-CXt specimens in tumors with partial response (p = 0.002 and 0.028) and minimal/non responding tumors (p = 0.062 and 0.002). In contrast a decrease in expression was observed in both observed groups for NOTCH1 (p = 0.072 and 0.001). IHC analysis of Notch2 revealed that cytoplasmic staining intensities of tumor cells increased significantly in all groups of CTx-treated patients (p = 0.016, 0.001 and 0.017) but not in non-CTx patients. Treatment of gastric cancer cell lines with 10 μM DAPT and 2 μM cisplatin led to a synergistic reduction of metabolic activity in comparison to the single drugs.

Conclusions: The comparison of mRNA expression between corresponding pre- and post-CTx specimens revealed alterations consistent with an enrichment of CTx-resistant tumor cells. Results of Notch2-IHC suggested an involvement of Notch-signaling in chemoresistance, which was further supported by the synergistic effect of cisplatin and the gamma-secretase inhibitor DAPT on cancer cells in vitro.

[339] The FGF Axis in Melanoma - FGF5 as Novel Player and Combination Strategies for Effective Targeting

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Introduction: Melanoma is a highly aggressive tumor with sharply rising incidence and high metastatic potential. Fibroblast growth factor 2 (FGF2, BFGF) and FGF-receptor (FGFR) 1 have been shown to contribute to melanoma cell motility but other key possible targeting strategies have received little attention so far. The aim of our study was to investigate the expression and function of FGFRs and FGFRs in melanoma cells and evaluate potential targeting approaches.

Materials and Methods: Expression of FGF and FGFR transcripts was assessed by expression array analysis and confirmed by quantitative PCR in melanoma cell models and normal melanocytes. FGFs was further investigated by immunohistochemistry in a tissue array. Genetic constructs and FGFR-specific tyrosine kinase inhibitors were used to augment or silence FGF-activated signaling pathways. The effects of altered FGF signals on melanoma growth were assessed in multiple functional in vitro assays and SCID mouse xenografts. Impact on signal transduction was analysed by immunoblotting using phosphorylation site-specific antibodies.

Results: Besides FGFR1 and FGFR4, 4, FGF5 was highly expressed in a subset of cell lines and was also detected in tissue samples. Ectopic expression of FGF5 had no effect on cell proliferation in vitro but increased clonogenic survival and tumor growth in vivo. In contrast, dominant-negative FGFR1 or FGFR4, but not FGFR3, inhibited melanoma cell growth in vitro and in vivo and induced apoptosis. Likewise, FGFR-directed tyrosine kinase inhibitors impaired growth of melanoma cells. FGFR inhibitors showed mostly additive or antagonistic effects when combined with the chemotherapeutic agent dacarbazine or the bispahosphonate zoledronate, but demonstrated synergistic activity upon combination with the BRAF inhibitor vemurafenib, the multi kinase inhibitor sorafenib or the EGFR inhibitor erlotinib.

Conclusion: Our data suggest that FGFs including FGF5 are important mediators of tumor growth in melanoma. Targeting FGFR may represent an important approach for therapeutic interference, especially in combination with inhibition of mutated BRAF or other oncogenic kinases.

[839] Nitric Oxide Suppresses the Expression of CXCL10 and Leads to the Poor Outcome of Stage III Malignant Melanoma

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Increasing evidences are suggesting that melanoma is heterogeneous disease in its molecular biology, etiology, and epidemiology. Among them, we previously reported that the expression of inducible nitric-oxide synthase (iNOS) in melanoma tumor cells is strongly correlating with poor patient survival. Therefore, we are hypothesizing that nitric oxide (NO) produced by iNOS is a key molecule in melanoma inflammatory tumor microenvironment of poor outcome. To gain further insight into the role of NO and iNOS in the melanoma inflammatory tumor microenvironment, we performed PCR array related to inflammatory and autoimmunity gene on a series of Stage III melanoma lymph node metastases samples. In the comparison of iNOS negative and iNOS positive patients tumor samples, expression of CXC Chemokine ligand 10 (CXCL10) was significantly upregulated in iNOS negative patient group. Expression of CXCL10 was also confirmed in immunohistochemical analysis of strong CXCL10 expression was observed in the favorable prognosis compared to low CXCL10 staining group. In the in vitro analysis, treating iNOS negative and CXCL10 positive melanoma cell line with NO donor, S-nitroso-N-acetyl-L-penicillamine suppressed the expression of CXCL10 in melanoma. Furthermore, in the analysis of the cultured media, quenching NO from iNOS positive cell line treated with NO scavenger, 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxide (cPTIO) showed the effect of NO in the differential gene expression with inclusion of expression of CXCL10 compared to non treated cell. Supernatants of cPTIO treated melanoma cells promoted the migration of plasmacytoid dendritic cell and it was diminished when treated with CXCL10 neutralizing antibody. CXCL10 is reported to play the role in the recruitment of CDE T, NK cells, and NK cells into the tumor microenvironment as well as antagonizing the activities of angiogenic factors and considered as an anti-tumorogenic chemokine. Our study suggests that production of NO by iNOS inhibits the expression of CXCL10 in melanoma and compromises the pro-tumorogenic tumor microenvironment. Inhibition of NO may render the tumor microenvironment to anti-tumorogenic status and iNOS could be considered as a target in the treatment of melanoma.

[840] MET Overexpression in Subtypes of Invasive Breast Cancer - A Potential Therapeutic Target for Basal-like Breast Cancer

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Background: Basal-like breast cancer (BLBC) is a clinically challenging subtype with this subtype harboring a high level of HER2 expression and a lack of targeted therapies. MET is a member of the receptor tyrosine kinase family, and recent evidence identified a role for MET in the induction of mammary mammary tumors with characteristics of human BLBC. In this study,
we evaluated MET expression in 5 subtypes of breast cancers and assessed MET as a potential therapeutic target for BLBC. We also evaluated the efficacy of MET inhibitor in BLBC cell lines.

**Material and Methods:** MET protein expression was evaluated by immunohistochemistry on tissue microarray samples from 924 breast cancer patients including luminal A (474 cases), luminal B (118 cases), HER2 (111 cases), BLBC (134 cases), and quintuple negative breast cancer (QNBC, 87 cases) subtypes, defined by expression status of estrogen receptor (ER), progesterone receptor (PR), epithelial growth factor receptor (EGFR), cytokeratin (CK) 5/6, and amplification of HER2 on FISH. The effect of MET inhibitor was monitored by multiplex analysis. MET expression in cell lines is measured using qRT-PCR, immunoblotting, and immunohistochemistry.

**Results:** The immunohistochemistry-based analysis showed that 55 (6.0%) cases overexpressed MET among 924 breast cancer patients. BLBC was the subtype showing the highest relationship with MET overexpression (28/134, 20.9%, P < 0.001) among 5 subtypes. MET was also significantly associated with absence of ER (P < 0.001) and PR (P < 0.001), expression of EGFR (P < 0.001) and CK 5/6 (P < 0.001), and treatment of adjuvant chemotherapy (P = 0.034). In breast cancer cell lines, MET expression was relatively high in BLBC cell lines. In these cell lines, depletion of MET using siRNA led decreased cell proliferation. MET inhibitor treatment resulted in decreased cell viability.

**Conclusions:** MET expression was highly correlated with BLBC, and MET inhibitor showed inhibited activity in BLBC cell lines. MET may be a potential therapeutic target for BLBC.

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**[41]** Non-invasive Imaging of Response to MEK Inhibition With Selumetinib (AZD6244, ARRY-142886) in a Human Colorectal Cancer Xenograft Using Diffusion-Weighted MRI


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**Materials and Methods:** FLIP as a Critical Target for Vorinostat in Mesothelioma

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**Background:** Malignant pleural mesothelioma (MPM) is a rapidly fatal malignancy with a median survival of less than 12 months. The caspase 8 inhibitor FLIP is a major anti-apoptotic protein that is over-expressed in several cancer types, including mesothelioma, and has been linked to drug resistance. One of the main reasons for the failure of anti-cancer therapies is resistance to apoptosis. The results of the clinical trial of Vorinostat for patients with relapsed MPM were disappointing. However, no molecular stratification was conducted, and no subgroups that may have benefited were identified. In order to develop clinically relevant predictive biomarkers of response, understanding of the mechanisms involved in Vorinostat-induced apoptosis in MPM is essential.

**Materials and Methods:** The role of FLIP and caspase 8 in mediating the effects of Vorinostat in MPM was assessed using cell line and spheroid models. siRNA and overexpression approaches were used, and cell death was assessed by flow cytometry, Western blotting and clonogenic assays.

**Results:** Vorinostat potently down-regulated FLIP expression in a panel of MPM cell lines (n = 7). Vorinostat-induced apoptosis was found to be highly caspase 8-dependent in 5/7 MPM cell lines examined, and stable FLIP overexpression inhibited Vorinostat-induced apoptosis. Importantly, FLIP down-regulation was a sufficient death signal, as RNAi-mediated FLIP silencing also activated caspase 8 and induced apoptosis. In addition, Vorinostat-induced FLIP down-regulation was observed also in 3D model of MPM. Moreover, Vorinostat was found to enhance cisplatin- and rTRAIL-induced death in a FLIP-dependent manner. Analysis of MPM patient samples demonstrated significant inter-patient variations in c-FLIP and caspase 8 expression.

**Conclusions:** These results indicate that FLIP is a major target for Vorinostat in MPM and identifies FLIP, caspase 8 and associated signaling molecules as candidate pharmacodynamic and/or predictive biomarkers for Vorinostat in this disease.

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**[53]** Tenasin in Breast Cancer

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Tenasin-C (TNC) is an extracellular hexameric matrix glycoprotein consisting of four domains (assembly domain, several EGF-L repeats, FNIII-L repeats, and which precede tumour shrinkage. Our data support the use of ADC as a non-invasive pharmacodynamic biomarker for early assessment of response to selumetinib and potentially other MEK-ERK1/2 signalling-targeted therapies during clinical trials.

This study highlights changes in ADC that follow treatment with a MEK inhibitor, and which precede tumour shrinkage. Our data support the use of ADC as a non-invasive pharmacodynamic biomarker for early assessment of response to selumetinib and potentially other MEK-ERK1/2 signalling-targeted therapies during clinical trials.
The Changes in MGMT Promoter Methylation Status in Primary and Recurrent Glioblastomas

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Background: To evaluate the mechanism of the development of therapeutic resistance after temozolomide treatment, we focused on changes in O6-methylguanine DNA methyltransferase (MGMT) and mismatch repair (MMR) between primary and recurrent glioblastomas.

Material and Methods: Tissue samples obtained from 24 paired histologically confirmed primary and recurrent adult glioblastoma patients who were initially treated with temozolomide were utilised for MGMT and MMR gene promoter methylation status and protein expression analysis using methylation-specific multiplex ligation probe amplification (MS-MLPA), methylation-specific polymerase chain reaction (MSP) and immunohistochemical (IHC) staining.

Results: There was a significant decrease in the methylation ratio of the MGMT promoter determined by MS-MLPA, which was not detectable with MSP, and MGMT protein expression changes were not remarkable. However, there was no epigenetic variability in MMR genes, and a relatively homogeneous expression of MMR proteins was observed in primary and recurrent tumors. Conclusions: We conclude that the development of reduced methylation in the MGMT promoter is one of the mechanisms for acquiring therapeutic resistance after temozolomide treatment in glioblastomas.

Inactivation of p16INK4a and Bim in Childhood B Non-Hodgkin Lymphomas

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Introduction: B non-Hodgkin’s lymphomas (B-NHL) are a group of aggressive childhood neoplasms comprising Burkitt lymphoma (BL) (65%) and Diffuse Large B Cell lymphoma (DLBCL) (24%) subtypes. Epigenetic alterations such as promoter methylation have been associated with prognosis of many types of cancers. However, little is known regarding the role of methylation in the pathogenesis of childhood B-NHL (BL or DLBCL), methylation-specific polymerase chain reaction (MSP) and Bim expression.

Objectives: The aim of this study was to evaluate the p16INK4a and Bim methylation in tumor samples of pediatric B-NHL and analyze the correlation between methylation and protein expression levels. Additionally, we assessed the association between Epstein-Barr virus (EBV)-positive tumors and aberrant methylation and the impact of methylation in the clinical outcome.

Material and Methods: Paraffin-embedded tumor samples from 80 pediatric patients diagnosed at the Brazilian National Cancer, Rio de Janeiro Brazil were evaluated. Protein expressions were analyzed by immunohistochemical staining and the quantification of DNA methylation was performed by using MSP after bisulfite conversion. Chi-square test, Kaplan Meier and log rank tests were used for statistical analyzes.

Results and Discussion: A total of 68 (85%) patients were diagnosed with BL and 12 (15%) with DLBCL. EBV was detected in 59% of tumor specimens. Overall, loss of p16INK4a expression was found in 50% of tumors and about 30% showed increased expression levels. Abrupt methylation of the p16INK4a gene was detected in about 70% of cases. Moreover, we found an association between DNA hypermethylation and decreased expression of p16INK4a (p = 0.022). Immunochemistry showed loss of Bim expression in 60% of tumors, whereas 14% showed a low expression, and only 16% of patients displayed high protein levels. Bim gene methylation was detected in 40% of the samples, with the majority (62%) hypomethylated and it was associated with the loss of protein expression. Recently, it has been reported that some proteins from EBV are capable of promoting the methylation of p16INK4a and Bim genes. No statistically significant association was found between Bim gene methylation and EBV-positive tumors (p = 0.188), however, this association was observed for p16INK4a (p = 0.011). On the other hand, p16INK4a and Bim methylation had no impact on the outcome (p = 0.423 and 0.697, respectively).

Conclusions: Inactivation of p16INK4a and Bim genes appears to play an important role in the childhood B-NHL pathogenesis. The presence of p16INK4a methylation in B-NHL was associated with loss of p16INK4a protein and EBV might be involved in its regulation. Further data also suggest loss of p16INK4a and Bim protein expressions have no impact on the outcome.


Inhibition of Focal Adhesion Kinase in Combination With Bevacizumab Reduces the Rate of Tumor Revascularization and Increases Survival in a Pre-clinical Model of Basal Breast Cancer

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Introduction: Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that provides a critical hub for signalling from at least four different classes of cellular signaling mechanisms including growth factor receptors, GPCRs, integrins and mechanical stress forces. By temporal and spatial integration of signals from these sources, FAK plays a central role in cell migration, invasion and proliferation; processes vital for tumorigenesis. The significance of FAK to the function of signal transduction pathways provides a strong rationale for the combination of FAK inhibitors with other targeted agents to achieve improved efficacy against a range of cancers. Others have demonstrated the importance of FAK in angiogenesis and therefore combining a FAK with anti-VEGF agents is an attractive strategy for this approach.

Materials and Methods: CTx-0294945 is an orally bioavailable small molecule ATP-competitive inhibitor of focal adhesion kinase (IC50 = 2.13 nM), which exhibits high selectivity against a diverse panel of 125 kinases including the closely related Pyk2. CTx-0294945 inhibits autophosphorylation of 397Y-FAK in MDA-MB-231 cells with an IC50 of ~7 nM and exhibits low cellular toxicity. To assess the co-administration of CTx-0294945 with bevacizumab (bev), mice were injected orthotopically with MDA-MB-231 cells (104). After 14 days, when tumors were palpable, mice were randomized into 4 groups and dosing commenced. The groups were treated with CTx-0294945, bev, CTx-0294945 and bev vehicle. Tumor growth was monitored and animals culled when the size of the tumors reached ethical end point (1000 mm3).

Results and Discussion: Tumor growth in the bev only and the CTx-0294945 + bev arms was significantly inhibited (75% and 88% TGI respectively) compared to control and CTx-0294945 arms. After harvest of control and CTx-0294945 cohorts, treatment of the other groups was stopped and tumor growth allowed to progress. After an additional 14 days the experiment was terminated when the bev group reached ethical end point; however at this time the average size of the tumors in the CTx-0294945 + bev cohort was still only 562 mm3. Histological analysis of tumor sections taken after harvest indicates that tumor re-vascularization was significantly reduced by the combination treatment.

Conclusions: Our data suggest the potential utility of combining the FAK inhibitor with bevacizumab to prevent tumour progression and enhance the durability of response.

Identification of a Biomarker Signature to Predict the Need for Chemotherapy in Patients With Hormone Receptor Positive Breast Cancer

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Background: Breast cancer has been recognized as a heterogeneous disease that consists of different intrinsic subtypes of which luminal (hormone receptor positive) breast cancer presents the largest group with 70–80% of newly diagnosed breast cancer patients. To assess the need for chemotherapy, patients suffering from clinically more aggressive luminal subtypes need to be identified. However, treatment decisions are often difficult since an intermediate risk of cancer progression is reported for the majority of patients with luminal breast cancer which results in over- or undertreatment.

Material and Methods: A tumor set consisting of 20 high grade and 20 low grade tumors were probed by targeted profiling using reverse phase protein arrays (RPPA) for >150 proteins and phosphoproteins playing a central role in cancer and cancer-relevant signaling. The resulting quantitative data was analyzed using a combination of different statistical classification methods (SVM, PAM, and random forest). The abundance of the five top candidates was subsequently analyzed for 80 tumors with an intermediate risk of cancer progression.

Results: This approach identified consistently a protein signature that includes proliferation markers such as MCM6, TOP2A, PTP4A3, and the nucleoside diphosphate kinase (NME/NDKA) which has previously been associated with the maintenance of chromosomal integrity. All proteins are upregulated in high risk tumors, and apparently present different molecular
subtypes of aggressive luminal tumors. Another protein candidate, caveolin-1, was significantly downregulated in high risk tumors in line with its known role in sabotage of the mitochondrial shift and tumor growth. Vice versa, low risk tumors express low levels of MKI67, TOP2A, RPS6 and NEMD/NDKBA but high amounts of caveolin-1. Eighty tumors identified with an intermediate risk of cancer progression showed standard histopathological characteristics of a high risk group when analyzed for the abundance of MKI67, TOP2A, RPS6 and NEMD/NDKBA and caveolin-1. The 5-protein marker was subsequently validated on tissue microarrays.

**Conclusions:** The proteomic signature consisting of caveolin-1, RPS6, NEMD/NDKBA, MKI67, and TOP2A is of potential value for therapeutic decision making.

### [E-cadherin and Estrogen Receptor (ER) Alpha and Beta1 Protein Expression Difference Between OSE and Oviduct Epithelium in Older Mice – Implications for Origin of Ovarian Epithelial Cancer](http://example.com)

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**Introduction:** Ovarian epithelial cancer (OEC) affects mainly older women and has been linked epidemiologically to hormone replacement therapy. OEC is thought to originate from ovarian surface epithelium (OSE), however oviduct epithelium, derived from the same embryonic tissue, has been suggested as an alternative site of origin. In OSE, decreases in ERb1 expression and upregulation of the cell adhesion molecule E-cadherin, appear to represent critical steps in developing OEC. Recently, we reported that normal ER expression and co-expression patterns differed within and between OSE cells in older mice according to cell shape, and that estradiol caused sizable downregulation of ERb1. Upregulation of E-cadherin in OSE has also been reported after a single estradiol treatment. In the present study using older mice, we employed multiple label immunofluorescence and confocal microscopy to compare normal ERa/b1 and E-cadherin/ERb1 protein expression patterns for oviduct epithelium with OSE, and to document the effect of estradiol on oviduct ER.

**Materials and Methods:** Swiss Webster mice (7-10 month old, n = 4/group) received subcutaneous injections of estradiol valerate (EV) 10 μg/kg body weight in castor oil (CO). Diestrous controls had CO alone. Mice were cycled 48 hours later and ovary and oviduct removed. Immunohistochemical localization by light microscopy was with polyclonal antibody directed against ERa, and monoclonal antibodies directed against ERb1 and E-cadherin. Co-localization of ERa/b subtype and E-cadherin/ERb1 protein expression patterns for oviduct epithelium with OSE, and to document the effect of estradiol on oviduct ER.

**Results and Discussion:** Whereas light microscopy showed strong nuclear stain for ERa in both oviduct and OSE, oviduct had greater staining intensity for ERb. Exposure of oviduct epithelium to estrogen resulted in reduced expression for ERa but little change to ERb. Double and triple immunofluorescence label visualized by confocal microscopy showed nuclear and cytoplasmic expression of both ER in OSE evident by light microscopy but not found in oviduct using this method. ERb localized predominantly to nuclei in oviduct epithelia however, a feature of columnar-shaped OSE cells. Co-localization of ER in oviduct was infrequent, aligning also with expression patterns for columnar, but not cuboidal, OSE. ERb expression relative to ERa appeared greater in oviduct. E-cadherin showed strong stain in oviduct delineating plasma membranes, but was seen only in columnar OSE. Co-localization of E-cadherin with ERb was not a feature of oviduct but there was some evidence for co-localization in the cytoplasm of columnar OSE cells.

**Conclusion:** ER and E-cadherin expression patterns differ between OSE and oviduct. ERb expression appears independent of E-cadherin in oviduct but not in OSE. These results may have implications for the genesis of OEC.

### [The Role of Bmi-1 Gene in Primary Myelodysplastic Syndrome – Correlation With Cytogenetics, Cellular and Clinical Features](http://example.com)

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Primary myelodysplastic syndrome (MDS) comprises a heterogeneous group of hematopoietic stem cell clonal disease. It is characterized by an inefficient hematopoietic progenitor cell function, chemoresistant dysplasia in bone marrow cells and peripheral blood cytopenias. Around 10–40% of the cases evolve to acute myeloid leukemia (AML). The apparent paradox of hypercellular bone marrow and peripheral blood cytopenias due to the presence of increased rates of apoptosis in bone marrow cells has been shown to be due to enhanced programmed cell death. However, there is controversy regarding apoptosis occurs only in progenitors cells or cells which have entered the cell proliferation program and if there is some correlation with the presence of chromosomal abnormalities. Cytogenetic analysis plays an important role in the MDS diagnosis, however, there are cases with normal karyotype rendering the recognition of independent molecular markers to aid diagnosis. It was recently demonstrated the involvement of the Bmi-1 gene process of proliferation and hematopoietic stem cell self-renewal. Some studies have shown a high expression of Bmi-1 gene in hematological malignancies. In MDS there are few studies suggesting the Bmi-1 gene may be a new molecular marker of progression to AML, however, there are no studies showing correlation between expression of Bmi-1 gene and the presence of specific chromosomal changes. The aim of this study was correlate the expression pattern of Bmi-1 gene with chromosomal abnormalities, cellular aspects and clinical features, relating with patients prognosis. Chromosomal analysis has been performed by classical (GTO banding) and molecular cytogenetic (FISH) techniques. For analysis of apoptosis using flow cytometry we labeled bone marrow cells with annexin V and specific antibodies for cells Lineages. The gene expression analysis was performed by the method of real-time PCR. Our results showed that the chromosome pattern in primary MDS is mainly characterized by partial and complete loss of chromosome. Regarding the study of apoptosis, higher rates were found in early stages when compared with more advanced disease. Cells committed with cellular differentiation process appears to be the primary target of apoptotic process. Bmi-1 gene showed to be more expressed in patients with primary MDS when compared with normal individuals (bone marrow donors). When compared with the levels of apoptosis was possible to observe that when apoptosis level is high the expression level of Bmi-1 gene is low, and when apoptosis level is low the expression level of Bmi-1 gene is higher. Also it was observed an association between higher expression of Bmi-1 gene and alterations of chromosome 11. Our results suggest that Bmi-1 gene is associated with progression of MDS to AML, being a possible marker for leukemia transformation.

**Supported by:** FAPERJ; CNPq Ministry of Health

### [Autoptopy Inhibition Sensitive Mantle Cell Lymphoma Cells to Everolimus](http://example.com)

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**Background:** Mantle cell lymphoma (MCL) is an aggressive B-lymphoid neoplasm with poor response to conventional chemotherapy and short survival. The phosphatidylinositol 3-kinase/Akt/mTOR survival pathway is constitutively activated in MCL cells, thereby making the mTOR inhibition an attractive therapeutic strategy. The first clinical studies of everolimus (RAD001), an mTOR inhibitor, in relapsed MCL patients have reported a significant response. Our aim was to analyze the mechanism related to everolimus resistance/ sensitivity in MCL cells.

**Material and Methods:** Sensitivity to everolimus was analyzed in MCL cell lines and primary MCL cells. Everolimus mechanism of action was determined by flow cytometry, and western blot. Particularly, autoptopy was studied by LC-MS/MS expression, autophagy detection by flow cytometry, and siRNA-mediated gene silencing.

**Results:** Everolimus exerted antitumoral effect on MCL cells while sparing normal cells. In MCL cell lines with high expression of both ER in oviduct epithelium, a finding also observed in oviduct using this method. ERb localized predominantly to nuclei in oviduct epithelium however, a feature of columnar-shaped OSE cells. Co-localization of ER in OSE was infrequent, aligning also with expression patterns for columnar, but not cuboidal, OSE. ERb expression relative to ERa appeared greater in oviduct. E-cadherin showed strong stain in oviduct delineating plasma membranes, but was seen only in columnar OSE. Co-localization of E-cadherin with ERb was not a feature of oviduct but there was some evidence for co-localization in the cytoplasm of columnar OSE cells.

**Conclusion:** ER and E-cadherin expression patterns differ between OSE and oviduct. ERb expression appears independent of E-cadherin in oviduct but not in OSE. These results may have implications for the genesis of OEC.

### [Top Down LC-MALDI Discovery of Cu/Zn SOD as Potential Biomarker for Inherent Chemoresistance](http://example.com)

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1. Indumed GmbH, Biomarker Discovery, Hamburg, Germany
2. Top Down proteomics approach, we identified the Cu/Zn SOD to be up regulated in chemoresistant cell cultures. Therefore, we further validated the Cu/Zn SOD as biomarker candidate.**
**Gene Expression Profiling of Advanced Rectal Tumor Patients Reveals a Molecular Signature Related to Response to Neoadjuvant Chemoradiation**

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**Introduction:** To date, none of the identified signatures or molecular markers related to response to pre-operative chemoradiation (CRT) in locally advanced rectal cancer (LARC) has been successfully validated as a diagnostic or prognostic tool applicable to routine clinical practice.

**Material and Methods:** Using Human WG CodeLink bioarrays, we established the expression profiling of 27 LARC without metastasis to gain insights into the molecular signatures associated with response to treatment after CRT. Tumor tissue biopsies and peripheral blood samples were obtained before CRT. Significant genes were found using Significance Analysis of Microarrays (SAM) (Real-time quantitative RT-PCR (RT-qPCR)) and FISH (fluorescence in situ hybridization) were used to confirm the results from the arrays. Results were correlated with Mandard's tumor regression grade (TRG): TRG1 and 2 being responders and TRG 3 to 5 Non-Responders.

**Results and Discussion:** The SAM method identified the more significant (adjusted p <0.05) differentially expressed genes between treatment Responder (n = 11) and Non-Responders (n = 16) LARC patients. All genes presented higher expression levels in Responder LARC samples. 257 genes were differentially expressed between the two subgroups of tumor sample patients. They included a broad range of genes involved in cell-cycle control and DNA synthesis, such as MAPK9, c-MYC, POLA, POLR2K, RRM1, MCM3, GNG4, and COASY, suggesting that Responder patients could have a higher proliferative rate than do Non-Responder. Instead 6 genes (CIR, PRDM2, CAPG, FAZ, NUP2L1, and ZFP36) were differentially expressed between peripheral mononuclear cells of BCs from Responder and Non-responders LARC patients to CRT. None of these genes were over-expressed in Responder tissue samples.

**Conclusion:** Our results reflect the value of gene expression profiling to gain insight about the molecular pathways involved in the response to treatment of LARC patients.

**Pharmacogenomic Profiling of Drugs in Cancer Cells Identifies Novel Biomarkers of Sensitivity**

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Targeted molecular therapies that disrupt specific intracellular signaling pathways are increasingly used for the treatment of cancer. Accumulating evidence indicate that clinical responses of patients to anti-cancer therapies are often correlated with specific tumour genotypes. To identify new biomarkers of drug sensitivity, we have systematically screened a large panel of cancer cell lines with 130 anti-cancer drugs and correlated drug sensitivity with genomic features. We find that the majority of cancer genes are associated with the cellular response to cancer drugs. As well as confirming many known drug-sensitizing genotypes (e.g. sensitivity to BRAF inhibitors in BRAF-mutant melanoma) we have identified many new gene-drug associations that may be useful as biomarkers of drug sensitivity. Notably, we identified an unexpected relationship between specific gene rearrangements (e.g. SWCNH fusion gene and sensitivity to inhibitors of PARP). Our work demonstrates the utility of pharmacogenomic profiling in cancer cell lines as a discovery platform to identify novel biomarkers to guide cancer therapeutic strategies.

**Identifying Tumour Vascular Connectivity With MRI and Independent Component Analysis**

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**Introduction:** Hypoxia within solid tumours is associated with impaired chemotherapy and radiotherapy response, as well as promoting metastasis and a more aggressive phenotype [1]. Acute, cyclical changes in tumour oxygenation have been measured using blood oxygen level dependent (BOLD) magnetic resonance imaging, and are thought to be due to microregional blood flow instabilities [1]. Independent component analysis (ICA) is a computational technique that is routinely used in the brain to identify patterns of neural activation at rest [2]. Here we use ICA to investigate the spatial connectivity of transient fluctuations in tumour oxygenation status, and look for associations between these and regions that are functionally perfused (i.e. respond to a hypercapnic/hyperoxia gas challenge).

**Materials and Methods:** Nude mice bearing SW1222 (n = 4) or LS174T (n = 3) colorectal carcinomas (~1 cm³ volume) were imaged in a 9.4T experimental MRI system, using a multi-slice, multi-gradient echo sequence. Two 70 minute scans were performed. The first one aimed to evaluate resting state spontaneous fluctuations in the BOLD MRI signal (R_2^*) in the tumour. The second one aimed to assess the response to hyperoxia/hypercapnia gas challenges, following air and carbogen (95% O₂, 5% CO₂) breathing.

**Results and Discussion:** Resting state: Variations in R_2^* were clearly identified in standard deviation maps of the tumour during resting state. No such variations were observed in normal muscle tissue. Furthermore, ICA maps of resting state revealed regions of common coherent oscillatory patterns. These maps show additional information over and above resting state maps, as we observed regions likely to have anti-correlated temporal patterns that may represent phenomena of intra-tumour vascular steal or a delay in the delivery of blood between two functionally connected regions.

**Conclusions:** ICA of resting state tumour data identified clusters of voxels with similar temporal characteristics, suggesting regions of comparable hemodynamic functionality. Moreover, these regions were found to be functionally perfused, as assessed by the gas challenge. This is the first reported application of ICA in this context, and although further measurements are necessary to accurately identify the observed clustering effects, it opens the possibility of using ICA as an approach for further investigating tumour vascular connectivity.

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**Reference(s)**

provide well characterised phenotypes to measure the biomarker potential of miRNAs. The aim of this study was to identify systemic miRNAs differentially expressed in luminal A (ER+PR+HER2/neu)- breast cancer, and to study their effectiveness as oncologic biomarkers in the clinical setting.

**Material and Method:** Whole blood samples were prospectively collected from women with luminal A breast cancer (n = 57) and healthy controls (n = 57). RNA was extracted, reverse transcribed and a Taqman Low-Density Array (TLDA, microarray) conducted on a test cohort (10 Luminal A; 10 Control). Differentially expressed miRNAs were identified using Artificial Neural Networks (ANN). Expression of specific miRNAs was validated using QP-PCR on an independent cohort (n = 47 Luminal A; n = 47 Control). Results were analysed using QBase and MiniLab V16.0.

**Results:** The TLDA performed on the test cohort identified 77 differentially expressed miRNAs from a panel of 867 miRNAs. Artificial Neural Networking highlighted seven miRNAs for further analysis (miR-A to miR-G). RQ-PCR quantification of expression of these seven candidate miRNAs in the validation cohort confirmed the biomarker potential of two miRNAs, miR-C and miR-E, were significantly under-expressed in the cancer group compared to the control group (p < 0.005 and p < 0.001 respectively). Circulating miR-C expression correlated with invasive tumour size (r = −0.592, p < 0.008). Furthermore, a combination of these two miRNAs provided a sensitivity and specificity for detection of Luminal A breast cancer of 70% and 65%, respectively (Area Under the Curve, AUC = 0.77).

**Conclusion:** This study provides over 70 miRNAs with altered systemic expression in Luminal A breast cancer, providing insight into the molecular profile underlying this subtype. Two novel miRNA oncologic biomarkers are identified, which may have a role in combination with mammography to provide accurate subtype-specific breast tumour detection.

**Note:** The specific miRNAs will be disclosed on the Poster.

### 550 Association of the Expression Profile of Prostate Cancer With TMPRSS2-ERG Status and Prognosis

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**Background:** The fusion gene TMPRSS2-ERG is harbored by almost the 50% of prostate cancer (PCa) patients. Furthermore, there are other genes with consistently altered expression in PCa including TMPRSS2-ERG. The clinical significance of this expression and of the rearrangement remains controversial nowadays.

The aim of the present study was to establish an expression profile according to the expression of these genes and otherwise to relate these data with the clinical and pathological parameters (CPP) used in the PCa management.

**Material and Methods:** A series 314 patients undergoing radical prostatectomy in the Fundacion Instituto Valenciano de Oncologia between 1996 and 2004 were studied. The median follow up was of 83.6 months (range: 2−159.8 mo). TMPRSS2-ERG fusion gene status was determined in fixed and formalin and paraffin embedded material using RT-PCR and FISH with commercial probes. In addition, we assessed the expression of ET4, PCA3, HEPSIN, PAR-2, GSTP1-1, AMACR, TMPRSS2- ERG and ETV1 by means quantitative RT-PCR. An unsupervised cluster analysis with the whole series of genes was performed. Expression profiles were associated with the CPP as well as with the biochemical (BPFS) and clinical progression-free survival (PFS) by the univariate log-rank survival test.

**Results:** The presence of TMPRSS2-ERG was detected in 47.1% of the analyzed cases. No significant association between the presence of TMPRSS2-ERG and BPFS and PFS was observed.

In an unsupervised clustering analysis the whole series of patients were classified into three groups according the expression level of the genes studied. However, the expression profiles were significantly related with the clinical and pathological stage (p = 0.006 and p = 0.003, respectively), the perineural infiltration (p = 0.028) and the TMPRSS2-ERG status (p < 0.001).

The first group consisted on PCa cases with a normal prostate-like phenotype, whereas the third group represented a more malignant phenotype characterized by higher clinical and pathological stages and the presence of perineural infiltration. This group was also constituted by many (78.3%) of cases harboring the TMPRSS2-ERG fusion gene. The intermediate group was comprised by 60% of the whole series, 43.7% of these cases carrying the fusion gene TMPRSS2-ERG.

**Conclusions:** PCA is indeed a very heterogeneous disease, from the point of view of its genetic diversity. The detection of TMPRSS2-ERG and the expression of the related genes can clarify the biology and the prognostic characteristics of this disease. The classification and characterization of PCa with molecular parameters would be useful in the clinical management of this disease.

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### 657 MicroRNA Predictor of Early Relapse in Advanced Stage Ovarian Cancer Patients

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**Background:** A major challenge in advanced-stage epithelial ovarian cancer (EOC) is prediction of chemoresistant relapse. In the attempt to identify a microRNA (miRNA) signature associated with early relapse in advanced-stage EOC patients we analyzed, by microarray profiling, patients’ set A (n = 55) and set B (n = 30) selected on the basis of time to relapse (TTR), followed by RT-qPCR validation on set C (n = 45) composed of consecutive cases unselected for clinical response. Our analyses identified a cluster of miRNA located on chrXq27.3, whose downregulation associated with shorter TTR (logrank, P = 0.00074, HR 2.44). The cluster was confirmed as an independent prognostic factor on publicly available datasets (Bagnoi & De Cecco et al, Oncotarget, 2011).

**Purposes:** In the perspective of a clinical grade predictive assay development we challenged the existence of a miRNA signature predictive of early relapse by gene list and algorithm refinement in an independent cohort.

**Materials and Methods:** The INT set C was microarray-profiled simultaneously with a set D, from CRO, comprising 111 consecutive advanced-stage EOC patients unselected for clinical response. The survival prediction was performed according to the Supervised Principal Component method (Bair & Tibshirani, PLoS Biology, 2004). A and B datasets, upon data normalization, batch effect correction and quality control, were assembled in an integrated dataset (A+B, n = 85). The miRNAs entered in the predictor were validated in silico on dataset A+B and by RT-qPCR on set C.

**Results:** A predictive model consisting of the weighted expression of 31 miRNAs was developed on set C and a threshold to stratify patients in low and high risk of early relapse was established (log-rank test statistic between risk groups, permutation P-value = 0.02). The predictive model was challenged and validated on set D (log-rank P = 2.01 × 10⁻⁶). When the predictor was applied to the dataset A+B the area under a ROC curve was 0.78 (95%CI: 0.675–0.861). Cross-validation of the 31 miRNAs by RT-qPCR resulted in the elimination of 3 miRNAs and in a substantial enrichment of the predictive value (log-rank test statistic between risk groups, permutation P-value = 0.0005).

**Conclusion:** Further refinements needed to build a clinical grade assay, such as the identification of potential redundancy inside the predictor using a non-negative matrix factorization and relevance network selection approach, are in progress.

### 554 Bayesian Adaptive Dose-escalation Designs in Practice

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**Background:** Much literature has been published showing that Bayesian adaptive dose-escalation designs are superior to traditional 3+3 escalation. However, while statistics are important to understand the considerable uncertainties inherent in phase I, they are only one component of good phase I designs. This may explain why real world applications of these designs are still rare and only a few examples exist, mainly due to operational challenges [1−3]. With almost 10 years of experience in Bayesian adaptive dose-finding, Novartis Oncology has unique expertise in implementing Bayesian adaptive dose-finding, including operational feasibility and recommendations for interaction with regulators and other stakeholders.
Material and Method: The reason why Bayesian adaptive dose-escalation outperform traditional 3+3 designs will be explained by use of examples from an actual study. This study will illustrate the standard Bayesian adaptive dose-escalation design used at Novartis Oncology, with an emphasis on the implementation in practice. Important milestones in preparation and conduct of the trial are highlighted, addressing concerns from clinicians. Experience from interaction with study sites, investigators, and health authorities will be shared. In order to enable researchers to implement such designs in practice, a roadmap with ‘step-by-step’ instructions will be presented.

Results and Discussion: The applications, together with instructions in the roadmap, will enable researchers to plan and conduct dose-escalation trials using a design [4,5] superior than traditional 3+3 escalation. Experience from interactions with different stakeholders will provide a base for the audience to adequately address the needs of the different parties involved (e.g., site personnel, investigators, regulatory agencies).

Conclusion: Bayesian adaptive dose-finding designs have clear benefits for both, patients and researchers. The use of these designs for dose-escalation is encouraged by health authorities [8], but their implementation in practice can be challenging. Even though they offer a much more appropriate and evidence-based way for dose-finding in Oncology, they require a considerable change in the paradigm of conducting dose-escalation.

Reference(s)

[559] TGF-β Inhibits TMEFF2 Expression in Glioma Cells
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Background: Tmeff2 is a protein with unclear function. It has been described that Tmeff2 promoter is silenced by hypermethylation in certain primary tumors. To date, no other mechanism regulating Tmeff2 expression has been described in tumor cells. In addition to other intracellular signaling pathways, the TGF-β pathway plays an important role in glioma genesis and development. Here we show that TGF-β regulates TMEFF2 expression in glioma cells by the classical pathway mediated by Smads and through c-Myc binding to TMEFF2 promoter.

Material and Methods: TMEFF2 expression was measured by RT-qPCR in clinical frozen samples or in human glioma cell lines. U87MG and U373MG cell lines were treated with TGF-β1 (100 pM) or vehicle. When used TGF-β1 receptor inhibitor SB431542 (2 μM, Tocris Bioscience) was used to pretreat cells.

Conclusion: TGF-β1 modifies the cells to silence TMEFF2 expression in glioma cells. Inhibition of TGF-β pathway by Smad2, Smad3, and c-Myc and synthetic molecules, such as SB431542, or C-Myc blocking on monocytes inhibited surface expression of the major histocompatibility complex (MHC) class I, CCRT, CD80, CD83 and CD86 on TAPCells, reduced interleukin (IL)-6 and tumor necrosis factor (TNF)-α gene expression, and also the TAPCells-mediated activation of melanoma-specific CD8+ T cells. Moreover, CD8+ T cells activation capacity was significantly reduced in TAPCells bearing the TLR4 Asp299Gly receptor. Finally, TAPCells-vaccinated stage IV melanoma patients showed the 7th 896A/G allele median survival rate compared with those carrying the TLR4 896 AA allele.

Conclusion: Our results indicate that TLR4 is a key receptor for the tumor lysis-mediated in vitro generation of clinically efficient antigen presenting cells. Further analysis of patients included in different vaccine protocols is necessary to definitively establishing a role for TLR4 polymorphism in immune responses.

[631] Nanofluidic Digital PCR Quantification of Multiple KRAS Mutations in Colorectal and Pancreatic Carcinoma
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Introduction: In order to use tumor-specific somatic mutations as biomarkers for clinical oncology, they must be detected in the presence of large excess of non-mutated DNA. We have hypothesized that quantification of KRAS mutant alleles as well as the presence of multiple alleles may provide additional information when compared to conventional mutation detection techniques. Aim: To assess the feasibility of nanofluidic digital PCR array platform as a quantitative and sensitive approach to simultaneously detect and quantify KRAS mutations in clinically relevant samples.

Material and Method: Two groups of patients (colorectal disease and pancreatic disease) were included: (i) 14 sporadic adenomas, 27 sporadic carcinomas and 5 controls; and (ii) 42 carcinomas of the exocrine pancreas, 10 carcinomas of the ampulla and 6 chronic pancreatitis. In 10 cases paired pancreatic juice was analyzed. KRAS mutations were assessed by conventional Real-Time PCR analysis using the Light Cycler® 480 (Roche Applied Science) and by the FluidigmTM Digital Array Chip and BioMark Platform. Both techniques used the same set of primers and probes. To determine the analytical sensitivity of the two techniques (nanofluidic digital PCR and conventional real-time PCR) we performed a serial reconstitution experiment with all known KRAS mutations at codon 12 (25%, 10%, 5%, 1%, 0.5%, 0.1% and 0.05%).

Results: Nanofluidic digital PCR was capable of detecting 0.05% to 0.1% of mutant alleles depending upon the variant analyzed. Colorectal: Nine of 14 (64%) adenomas were positive for KRAS mutations using conventional PCR while Digital PCR was able to increase this number.
Differentially Expressed MicroRNAs in Gastric Cancer-associated Myofibroblasts

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Introduction: MicroRNAs (miRNAs) modulate gene expression through translational repression and/or mRNA degradation and are deregulated in cancer cells. Stromal cells such as myofibroblasts are recognised to drive cancer progression but the mechanisms remain unclear. In this study, we have compared hsa-miR-181d miRNA expression in myofibroblasts derived from gastric tumours, adjacent and normal gastric tissues; the significance of a candidate miRNA for cell migration and proliferation was determined.

Materials and Methods: Primary myofibroblasts (n = 12) were generated from gastric cancers (CAM), adjacent non-neoplastic (ATM) and normal (NTM) gastric tissues. Ethical Committee permission was obtained from the relevant regulatory authority and informed written consent was obtained. MicroRNA expression was determined using locked nucleic acid (LNA) microarray (Exiqon) and subsequently validated using real-time PCR. Bioinformatic analysis on the differentially expressed miRNA datasets were performed using dChip and MetaCore pathway analysis software. Myofibroblasts and two gastric cancer cell lines (MKN45, AGS) were studied using Western blotting for WNT-3A and -5A, and immunocytochemistry for nuclear localisation of β-catenin; cell migration and proliferation were determined following WNT stimulation and knockdown of hsa-miR-181d.

Results: Principal component analysis (PCA) of global miRNA expression of CAMs and NTMs revealed two distinct groupings. qPCR validated the microarray data. There were 83 differentially expressed (p < 0.05) miRNAs in CAMs versus NTMs, and 106 in ATMs versus NTMs. Moreover, there were 36 differentially expressed (p < 0.05) miRNAs in CAMs versus their ATMs with the greatest difference in hsa-miR-181d. Network analysis revealed that these differentially abundance miRNAs were involved prominently in cell cycle regulation, WNT signalling and cell-matrix interaction. Furthermore, comparison of CAMs versus ATMs in individual patients revealed only WNT signalling as significantly deregulated in all patients. Increased β-catenin nuclear localisation and WNT-5A protein levels were observed in CAMs compared to their respective ATMs. WNT-5A protein was absent in gastric cancer cells. Recombinant WNT-3A and -5A stimulated migration and proliferation of CAMs, and migration of cancer cells. Conditioned medium from CAMs stimulated migration of both CAMs and AGS cells and was reduced after knockdown of hsa-miR-181d.

Conclusions: Deregulated miRNAs in CAMs are implicated in WNT signalling; hsa-miR-181d is increased in CAMs and plays a role in regulating cell migration.

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Differentially Expressed MicroRNAs in Gastric Cancer-associated Myofibroblasts

B-Raf, C-Kit and MGMT Molecular Evaluation on 316 Consecutive Patients (pts) Affected by Metastatic Melanoma (MM) and Receiving Different Treatment Combinations – Do Mutations Influence Outcome?

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Background: Advanced MM pts prognosis remains poor and it is not improved by standard treatments. Recent drugs such as Iplimumab and Vemufarnib, showed for the first time to impact on survival. B-Raf is a MAPK tyrosine-kinase mutated in about 62% of cutaneous MM, and C-Kit is a RTK mainly mutated in mucosal (39%), acral (38%) and sun damaged cutaneous(28%) melanomas. Finally, MGMT is a gene encoding for O6-methylguanine-DNA-methyltransferase, which is mutated in about 15% of melanomas and predicts Dacarbazine/Temozolomide response. Using available tissue samples from our Pathology Department, we looked at these molecular markers in order to test whether any of them could correlate with therapy response and survival.

Patients and Methods: Between January 2010 and December 2011, 316 chemo-naïve advanced MM pts underwent CT with different drug combinations, being molecularly evaluated on tissue blocks obtained for diagnostic. Treatment outcome was analyzed on Response Time to Progression and Overall Survival. Lately we explore any combination between the molecular markers, administered therapy and outcome.

Results: 316 pts had been evaluated at the analysis-time on available tumor tissue samples. B-Raf and c-Kit mutation analysis as well as methylation analysis for MGMT were performed showing 123/316 B-Raf, 12/210 c-Ki and 55/300 MGMT mutations. These molecular data have been correlated to pts outcome and will be presented and discussed.

Conclusions: Advanced melanoma pts show consistent mutations in B-Raf, c-Ki and MGMT genes. Here, we show a correlation between these molecular markers and response to therapy or survival.

SATB2 is an Independent Predictor of Outcome in Colorectal Cancer

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Background: SATB2 is a nuclear matrix-associated transcription factor that orchestrates gene expression in a tissue-specific manner by regulating higher order chromatin structure. Using antibody-based screening of 48 normal human tissues and 20 cancer types, SATB2 is identified as a protein almost exclusively expressed in gastrointestinal tissue. Differential expression of SATB2 was observed in colorectal cancer, with loss of expression occurring along the adenoma-carcinoma sequence also confirmed at the mRNA level in 10,533 colorectal cancer samples.

Materials and Methods: Knockdown of SATB2 expression in parental SW480 cells was done by siRNA and ectopic expression was carried out using pLenti6 vector. Cell viability assays and migration assays were performed. Tissue microarray and automated image analysis of SATB2 expression in colorectal cancers (n = 309) and (n = 290) was carried out to examine SATB2 expression and relationship of SATB2 with survival in independent cohorts. Gene set enrichment analysis was performed in two independent colorectal cancer cohorts (n > 500). Tissue microarray and automated image analysis of SATB2 expression in inflammatory bowel disease (n > 1000) cohort was examined.

Results: SATB2 expression was markedly decreased in a metastatic variant of SW480 colon cancer cells and siRNA knockdown of SATB2 expression in parental SW480 cells increased their growth and migratory capacity. Ectopic expression of SATB2 in the metastatic variant reversed the observed phenotype. SATB2 was demonstrated by multivariate Cox regression analysis to be an independent predictor of disease-specific survival (HR = 0.52, 95% CI 0.32–0.83, p = 0.006) and loss of SATB2 expression significantly correlated with poor differentiation (p = 0.001). SATB2 mRNA levels were examined in a second cohort (n = 290) and again, SATB2 was demonstrated to be an independent predictor of disease-specific survival (HR = 0.40, 95% CI 0.18–0.92, p = 0.031). Interestingly, in colorectal cancer patients, SATB2 levels significantly correlate with CD3+ T-cell infiltrates in the tumours (p = 0.006) and inversely correlate with COX2 expression (p = 0.019). Gene set enrichment analysis in two independent colorectal cancer cohorts (n = 500) revealed that SATB2 low tumours demonstrate altered immune signalling with significant increases in IFNg (p = 0.001), IL6 (p = 0.021) TFGb (p < 0.001). Furthermore, in patients with inflammatory bowel disease (n > 1000), we observed a significant correlation between loss of SATB2 expression and occurrence of future cancers (p = 0.013).

Conclusion: We postulate that SATB2 acts as a master regulator of the inflammatory response in the gut and loss of expression is significantly associated with the progression of colorectal cancer.

Bimarkers to Predict Brain Metastasis – Towards New Therapeutic Opportunities

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Background: It can be assumed that up to 30% of metastatic breast cancer patients will develop brain metastasis during the course of their disease. The increasing incidence of brain metastasis in cancer patients with otherwise...
well controlled systemic disease is a key research challenge, since the mean survival of these patients is seven months (Palmeiri et al. 2007).Coupling high resolution promoter analysis data we identified functional phenotypes that might increase brain metastasis risk, corresponding with a profile of proteins associated with the endoplasmic reticulum stress phenotype, which are markers of brain metastasis risk (Sanz et al. 2011).

Material and Methods: We have evaluated the expression of these brain metastasis markers by IHC in a multicentre assay with samples from three different Hospitals (n = 298, 84 with and 114 without metastasis after seven years). Follow up data was obtained by a medical diagnosis to assess the potential of these biomarkers to predict brain metastasis when already present in primary breast carcinomas.

Results and Discussion: The preliminary analysis of the data showed a correlation between three markers and brain metastasis development. We have tested the predictability of brain metastasis for each marker using a 2-sided Fisher exact test and summarized by calculating the sensitivity among tumors that developed metastasis, and specificity among tumors without metastasis, for strong positive values. We have calculated the positive and negative likelihood ratios as integrated predictive indexes, hence evaluating the area under the ROC curve. A multivariate analysis based on stepwise logistic regression retained GRP94, FN14 and Inhibit expression further increased the discrimination of brain metastasis development (aROC = 0.79, 95% CI 0.89–25.65).

Conclusion: The markers that we report here might help in the guidance of therapies in high risk subgroups breast cancer patients as triple negative and Her-2 positive, given the current cancer therapy development scenario where the global aim is to optimize results and minimize toxicity. Study supported by Ministerio de Sanidad y Consumo FIS P10/00057 and AVCRI-PPV033−08-BancSantanderwithFBGandUB.

A Highly Specific Cancer Target, Regulated Via Promoter Methylation

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Introduction: An ideal antigen for cancer immunotherapy should display restricted expression to tumor cells but absence in normal tissues, sparing normal cells from being targeted by immunotherapeutic treatments. One important limitation of cancer immunotherapy is the possible loss of the tumor specific antigen (TSA) in the tumor. A possible regulation of TSA expression might disclose possible mechanisms for their re-expression in tumors resulting in more efficient tumor eradication. Recently, we described isoform 2 of the tight junction molecule Claudin 18 (CLDN18.2) as a candidate for serous ovarian tumors and non-small cell lung cancer. Various cancer types, tight junctions are found to be disrupted, a characteristic associated with higher metastatic potential thus qualifying Claudin multi-gene family members as potent cancer candidates. Using in silico analysis, we identified another Claudin molecule (named GT512) as a possible TSA in three different cancer types. The regulation of GT512 is largely unknown. Recent studies using ChIP-seq identify binding sites for CTCF and TADs in the promoter of GT512. Both factors are able to exert regulation of gene expression in dependence of DNA-methylation. BORIS is known to be up-regulated in various cancer types, e.g. ovarian and lung cancer and regulates the expression of cancer-testis antigens such as NY-ESO1 and MAGE-A1. Here, we summarize our efforts to elucidate the epigenetic and transcriptional regulation of this GT512 in cancer.

Material and Methods: We analyzed the mRNA expression of GT512 in various cancer and normal tissues by quantitative RT-PCR. The methylation status of the promoter was analyzed using the demethylating agent 5-aza-2’-deoxycytidine, bisulfit sequencing and methylation-specific qPCR (mSPqPCR) in different human tumor cell lines, normal, and cancer tissues. RNA-mediated silencing of BORIS was performed and the change in expression level of GT512 was assessed by qRT-qPCR.

Results and Discussion: GT512 is highly and frequently expressed in human testicular, ovarian, and lung cancer but not in any normal tissue. The analyzed promoter region is hypomethylated in GT512 expressing tumor cell lines but hypermethylated in non-expressing tumor cell lines. Normal lung and ovary appear to be unmethylated whereas GT512 non-expressing tumors exhibit a higher methylation as GT512 expressing tumors. Silencing of BORIS resulted in a significant decrease of GT512 expression in the ovarian cancer cell line NIH-OVCAR3. Finally, we observe a significant correlation between BORIS and GT512 mRNA expression in ovarian cancer.

Conclusion: In this study, we introduce GT512 as a new cancer target for cancer immunotherapy. DNA-methylation of its promoter is involved in the regulation of GT512. Future studies aim to decipher the methylation status of the promoter in a larger cohort of ovarian and lung cancers as well as various normal tissues using MSqPCR. Furthermore, we will analyze in which extend BORIS is responsible for the distinct expression of GT512 in these cancer types.

Metronomic Chemotherapy Following the Maximum Tolerated Dose in a Sequential Chemo-switch Schedule is an Anti-angiogenic and Effective Strategy in a Pancreas Cancer Model

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Background: Metronomic chemotherapy, is based on more frequent and low-dose drug administrations compared to conventional schedule at the Maximum Tolerated Dose (MTD). This alternative schedule inhibits tumor growth primarily through anti-angiogenic mechanisms while reducing toxic side-effects. Metronomy represents an interesting alternative for a personalized cancer treatment administration strategy that could be given after the standard MTD treatment in an already suggested multi-targeted Chemo-switch schedule. In this work, we report the effectiveness of this new chemo-switch schedule in two distinct human pancreatic cancer models.

Material and Methods: Both models were orthotopic human pancreatic adenocarcinoma xenografts in nude mice. NP9 was originated after implantation of a pancreatic cancer cell line while TP11 was generated by a primary tumor from a patient.

Results: Mice were treated with Gemicibine on three different schedules: Metronomic (1 mg/kg/day, METG), the Maximum Tolerated Dose (100 mg/kg/day, MTDG) and a Chemo-switch schedule (MTDG and Chemo-switch schedule (MTDG dosing on days 0, 3, 6, 9 followed by the METG from day 12 until day 28, C-SG).

Results: In both pancreatic cancer models, metronomic treatment effect on established tumor was equivalent to MTDG administration reducing 40% tumor growth. Instead, the chemo-switch treatment had the most favorable effect compared with the other groups reaching the 80% of tumor growth inhibition. To study the anti-angiogenic effect of the C-SG schedule we performed another experiment comparing METG, C-SG and the combination schedule of MTDG followed by the monoclonal anti-VEGF receptor antibody DC101 (from day 12 until day 28). We found a similar effect on tumor growth inhibition in C-SG group and in the combination schedule of MTDG plus DC101. This effect correlated with a marked and significant decrease in CD 31 and laminin staining area (endothelial markers) in both groups.

Finally, while peritoneal metastases were observed in control and MTDG group, no dissemination was observed in the METG and C-SG groups.

Conclusions: These findings provide confirmation that a standard of care chemotherapy, followed by metronomic maintenance administration, named chemo-switch schedule is a potentially tractable clinical strategy with a demonstrated anti-angiogenic basis and a dissemination blocking role.

From Yeast Genetics to BRCA2 Tumorigenesis – Developing a New Functional Assay to Characterize Missense Variants

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Introduction: The tumor suppressor factor BRCA2 has been demonstrated to be involved in the maintenance genome integrity by affecting DNA double strand break repair. In this regard, BRCA2 mutant cells exhibit defective homologous recombination (HR) and hypersensitivity to DNA damaging agents. Mutations in BRCA2 contribute approximately 5% of breast and ovarian cancer cases that lead to the predisposition of women to early-onset breast and ovarian cancer, and account for 15% to 30% of familial breast cancer risk. In recent years, a large number of missense mutations, in-frame deletions and insertions in the BRCA2 gene have been reported as unclassified variants (UCVs), since their clinical significance to cancer risk has not been determined yet. As the yeast S. cerevisiae has been exploited to characterize mutations in tumor suppressor genes (BRCAT), we want to define if yeast is a good model for BRCA2 to characterize UCVs.

Materials and Methods: The human BRCA2 cDNA was cloned downstream the Ga1’ promoter and expressed in a diploid yeast strain. Then, we created
several BRCA2 point mutations in the CDNA which encoded mutant proteins related to familial cancer (10 variants that are not previously classified in other papers, G2748D as pathogenic control, M191ST and A295ST as neutral controls). As yeast is a very reliable system to study HR, we determined the effect of BRCA2wt and several mutants on yeast HR. Next we investigated if BRCA2 expression in yeast could affect the ability to form Rad52 and Rad52 foci after DNA damage induction with methyl methane sulfonate (MMS).

Results and Discussion: The expression of BRCA2 wt induces recombination in yeast. The G2748D variant, used as pathogenic control, has no effect. The neutral variants increase recombination although less significantly than wt BRCA2. This indicates that BRCA2 may get involved in the yeast HR pathway and, more importantly, the missense variants have a quite different phenotype when expressed in yeast. Indeed, this system seems to discriminate between neutral and probably pathogenic variants. Moreover, BRCA2 expression has no effects on Rad51 and Rad52 foci number after DNA damage, but increases the survival to MMS confirming a BRCA2 activity in yeast DNA damage repair pathways.

Conclusion: Our results indicated that yeast could be a promising tool to characterize the UCVs and to investigate the molecular basis of BRCA22 tumorigenesis.

[858] Risk Prediction of Tumor Relapse in Oral Cancer by Multi-parametric Marker Integration

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Background: Oral cavity cancers are the 7th tumor by diffusion worldwide and more than 90% are Oral Squamous Cell Carcinomas (OSCC). Late diagnosis and the aggressive nature of the disease cause tumor relapse in 25%-50% of cases. Despite different clinical and biomolecular prognostic factors have been reported, none of them show sufficient accuracy in discriminating relapsing versus non-relapsing patients. We have therefore elaborated an integrated platform for prospective prognostic classification of OSCC patients that comprehensively takes into account clinical parameters, radiological images, histopathological traits and gene expression patterns of their primary tumor lesions.

Methods: The study was performed on 129 enrolled OSCC patients surgically treated and followed up for at least 18 months. Clinical and imaging data were collected at the time of diagnosis and during follow-up; smoke, alcohol consumption and other risk factors were noted. Surgical specimens of primary lesions were collected and processed for histological diagnoses and classified according to the TNM staging system. Moreover, fresh tumor tissue samples were immediately frozen and used to extract DNA and RNA for molecular studies. Genome-wide expression profile was obtained by DNA microarray and the most representative genes were validated using qPCR by TaqMan Low Density Arrays. Pooled samples of normal epithelia of the oral cavity were used as a reference tissues in all molecular analyses.

Results: Through bioinformatic algorithms and dedicated software tools, gene expression profiles from relapsing and non-relapsing patients were compared and integrated with clinical, imaging and histopathological data to extract a multi-parametric platform for relapse prediction. This system identified as remarkable parameters N staging, perineural invasion, smoke, lymphoplasmacytic reaction, p53 stain, tumor thickness, extra tumor and extra nodal spreading, number of lymph nodes, site, texture and PHACTR1, SOD2, C17orf71, RPRM, CR1YA, ZNF205, AMDMD1, SLC5A12, C1orf67 as the most modulated genes.

Conclusions: Our analyses prove that multi-parametric evaluation of clinical and biological traits of OSCC patients at time of diagnosis and after surgical intervention on primary tumors may be exploitable for a prediction of higher risk of relapse with a superior accuracy of any currently reported system and open for the implementation of an individualized and more tailored management of OSCC patients.

[870] F10503L01, a Potential New Treatment for Melanoma – Preclinical Efficacy

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Background: Metastatic melanoma is one of the most aggressive tumours and with worst prognosis. Approximately 47,700 cases of melanoma are currently diagnosed per year in the USA and its incidence is increasing at the rate of 4.3% per year. FAES FARMA S.A. is about to complete the preclinical development of a molecule from its own R&D portfolio, F10503L01, which has shown a marked in vitro antitumor activity against solid tumour cell lines as well as in vivo animal models of melanoma.

Material and Methods: In vitro mechanistic studies on cell viability, cell cycle and relevant molecular targets mediating melanoma growth were evaluated in cultured cells treated with F10503L01. In in vivo experiments, male C57BL6/J or nude mice (12-14 wks-old, 3-5 per group) were injected with mouse melanoma B16 cells, which exhibit an aggressive growth and are widely used as a model to study cancer biology and therapeutics. Animals were inoculated with 2 x 106 cells in each flank and 24 h thereafter F10503L01 or vehicle was administered i.p. or i.v. under different protocols of timing and doses. For tumour growth measurement, animals received luciferase i.p. and luminescence was determined during isoflurane anesthesia using an IVIS CALIPER, which allows real-time analyses of tumour burden in live mice. In addition, tumour mass or survival at the end of the experiment was determined.

Results: Clear effects of F10503L01 over melanoma cells in vitro suggested a potential efficacy in in vivo melanoma models. In fact, F10503L01 exhibited a significant marked antitumour activity in the B16 melanoma tumour mouse model both after i.p. or i.v. administration. The antitumour efficacy involved a dose-dependent significant reduction in luminescence as well as in tumour mass. Kaplan–Meier analysis revealed a favourable effect on survival. In addition, analysis of side-effects on main organ function indicates a modest liver injury that rapidly recovers upon treatment discontinuation.

Conclusion: The new compound F10503L01 from FAES FARMA shows a potent antitumour effect in vivo and warrants further clinical investigation as a potential new anticancer agent. Using a broad panel of melanoma cells from different origins and with specific characteristics in terms of aggressiveness, nature of growth and tridimensional pattern of proliferation would provide the proof-of-principle and rationale for its further clinical evaluation.

[871] The Use of Automated Image-cytometry for Improved Non-invasive Bladder Cancer Detection by Multi-probe FISH Assay

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Background: UroVysion® multi-targeted fluorescence in situ hybridization (FISH) assay is a promising tool for detection of bladder cancer, however, the recommended targeting carries limitations and being a complex assay, signal evaluation is laborious. Our aim was to eliminate those drawbacks by using a user trainable automated microscopic workstation.

Material and Methods: To solve targeting problems chromogenim immunoperoxidase (cytokeratin-7) was performed and cells on slides were ‘sorted’ automatically (Zeiss, Axioplan II®; Melafer 4, Metacyte®) according to their immunostain related pixel intensities. Consecutive FISH analysis was performed manually, strictly on presorted nuclei. Analytical efficiency was determined using tumourous cell line (HT13-77) and healthy peripheral blood as controls; diagnostic efficiency was determined by testing the method on samples received from 42 patients. Parallel investigations of automated signal detection implied same workstations: morphometric parameters were: counterstain object threshold, min object area, max object area, max concavity depth, aspect ratio for nuclei detection and spot measurement area, min spot distance, min spot relative intensity for spot counting. Analytical efficiency was, again, determined using the above controls; clinical studies were performed on samples from 21 patients.

Results: The specificity of FISH-alone and targeted-FISH appeared 86% and 100%, respectively; while overall sensitivity was 80% and 93%, respectively. The ratio of FISH positive cells was proportionate with stage and grade, however, results were only significant applying targeted-FISH. Analytical and diagnostic accuracy of automated spot counting was comparable to that of manual approach (94.8% and 71% vs 97.9% and 76%, resp.). Accuracy increased with degree of dysplasia, thus, diagnostic sensitivity was poor in low-grade/stage cases. The system could not discriminate urothelial cells from others.

Conclusions: Immunophenotypic targeting raises accuracy of UroVysion and could lighten real tumor load. By applying the adequate antibody this method could also help identify tumor cells in ambiguous cases or provide clarification when other tumors are present. Automated spot counting appears feasible, but because it could also be affected by miss-targeting, our next aim is to combine the two methods, and create a comprehensively automated, high efficiency cell based diagnostic method for bladder cancer detection.
Acute STAT3 Activation in Colorectal Cancer Cells Following HDAC Inhibitor Treatment Results in Apoptosis Resistance

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Histone deacetylases (HDACs) can regulate both the expression and activity of a diverse range of proteins involved in tumour initiation and progression. The primary role for HDACs is in epigenetic regulation however they have also been shown to alter the activity of a range of proteins, such as transcription factors and proteins involved in cell proliferation and apoptosis through deacetylation. HDAC inhibitors are a new class of anti-cancer drug and are the subject of over 190 clinical trials at present. Pan-HDAC inhibitors, such as Vorinostat, have been shown to alter the activity of a range of proteins involved in cell proliferation and apoptosis through deacetylation. HDAC inhibitor treatment might represent a powerful strategy to increase survival, expansion and increase the overall survival in an ALK-positivelung carcinoma.

We generated transgenic (Tg) mice ectopically expressing human TFG- or EML4-ALK protein in lung epithelium under the control of the all trans retinoic acid receptor (ATRA) promoter and performed at least 3 immunization. To evaluate the generation of an immune response, we injected 0.5 mg of anti-CD25 i.p. at day 2 and day 5 after vaccination. To further increase anti-tumor immune response, we blocked T cell inhibitory molecules by treating mice with 400 µg of each anti-PD-1 and anti-PD-L1 antibodies.

Results and Discussion: TFG- or EML4-ALK mice develop tumor rapidly with high penetrance. ALK-DNA vaccination elicited a strong ALK-specific in vivo CTL response and increased the number of T lymphocytes infiltrating the tumor in ALK positive vaccinated mice. After vaccination, tumor progression was significantly delayed as vaccinated mice had a reduced number of neoplastic foci and smaller tumors, as compared to control mice. Remarkably, the survival rate of vaccinated mice was significantly higher than controls. Vaccination combined with the ALK kinase inhibitor TAE684 further delayed tumor recurrence and increased the overall survival. ALK vaccination increased intratumoral Treg cell frequency. TAE684 treatment, with the ALK inhibitor TAE684 at 25 mg/kg/day for 10 days and 10 mg/kg/day for 5 days. For Treg cell depletion, we injected 0.5 mg of anti-CD25 i.p. at day 2 and day 5 after vaccination. To further increase anti-tumor immune response, we blocked T cell inhibitory molecules by treating mice with 400 µg of each anti-PD-1 and anti-PD-L1 antibodies. Histology and immunohistochemistry were performed on different specimens with specific antibodies.

Results and Discussion: TFG- or EML4-ALK mice develop tumor rapidly with high penetrance. ALK-DNA vaccination elicited a strong ALK-specific in vivo CTL response and increased the number of T lymphocytes infiltrating the tumor in ALK positive vaccinated mice. After vaccination, tumor progression was significantly delayed as vaccinated mice had a reduced number of neoplastic foci and smaller tumors, as compared to control mice. Remarkably, the survival rate of vaccinated mice was significantly higher than controls. Vaccination combined with the ALK kinase inhibitor TAE684 further delayed tumor recurrence and increased the overall survival. ALK vaccination increased intratumoral Treg cell frequency. TAE684 treatment, with the ALK inhibitor TAE684 at 25 mg/kg/day for 10 days and 10 mg/kg/day for 5 days. For Treg cell depletion, we injected 0.5 mg of anti-CD25 i.p. at day 2 and day 5 after vaccination. To further increase anti-tumor immune response, we blocked T cell inhibitory molecules by treating mice with 400 µg of each anti-PD-1 and anti-PD-L1 antibodies. Histology and immunohistochemistry were performed on different specimens with specific antibodies.

Specific high affinity receptor targeting in SLNB has now been shown to be a key staging tool for BC-MA-SCC and appears to be a significant improvement over other SLNB agents. Current literature indicates that the SLNB procedure not only defines disease-dependent outcome, but when conducted effectively, can be used to significantly mitigate additional surgical morbidity. Tc99m-Tilmanocept provides significantly enhanced improvements in key factors affecting SLNB outcome. The impact of this improvement on staging of BC-MA-SCC and reduced morbidity as a whole may be clinically significant.

Non-invasive Monitoring of Metastatic Breast Cancer by Circulating Tumour DNA – a Comparison With Circulating Tumour Cells

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Background: In the management of metastatic breast cancer (MBC), monitoring of tumour response is crucial for determining the efficacy of treatment. Imaging is routinely used, but is often a poor indicator of dynamic clinical response. Circulating tumour cells (CTCs) have been extensively studied but dynamics of cell-free DNA carrying tumour-specific alterations (circulating tumour DNA, ctDNA) has only recently been demonstrated. In this study, we provided the first direct comparison of the dynamic changes of CTCs and ctDNA, in relation to medical imaging (CT scan), to compare their performances in monitoring the treatment of MBC patients.

Methods and Materials: Clinical samples were collected prospectively from women undergoing therapy for MBC. DNA from tumour tissues was analysed using targeted or whole genome sequencing to identify somatic genetic alterations, including point mutations and structural variants. Personalised digital PCR assays were designed to quantify ctDNA in plasma. CTCs were quantified at identical time points using the CellSearch® system. All imaging data were reviewed according to RECIST criteria in a blinded fashion.

Results: Concurrent CTC and ctDNA data were available from 16 women across 58 blood samples. Elevated CTCs (>5 cells/7.5 ml blood) and ctDNA were identified in 11 (69%) and 15 (94%) cases respectively. Rising ctDNA was identified months before progressive disease as identified by CTCs or imaging. Changes in ctDNA levels were also observed in a subset of women with no measurable disease using the other modalities. In some cases, ctDNA were measured by multiple somatic mutations and structural variants in parallel, enabling the assessment of clonal changes during treatment.

Conclusions: This study demonstrates that ctDNA provides the earliest measure of treatment response compared to both CTCs and imaging. The data also suggest that it has the potential to be used to directly monitor responses.
to targeted therapies and detect the emergence of resistant mutations during treatment. Further work such as multi-centre prospective clinical trials would be needed to evaluate its potential to be implemented clinically.

**Use of PARPi to Overcome Cisplatin Resistance in Testicular**

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**Introduction:** Testicular Germ Cell Tumours (TGCTs) are the most frequent type of cancer in young males. Nowadays, the high sensitivity of TGCTs to cisplatin (CDDP)-based chemotherapy, together with surgical procedures, leads to a very high cure rate. However, 15–20% of patients with disseminated disease will not achieve a complete long term response, resulting in therapeutic failure and, eventually, death of the patient. In testicular refractory tumours hyperactivation of DNA-damage repair mechanisms is thought to play an important role in the onset of resistance to platinum drugs. Poly(ADP-Ribose) Polymerases (PARP) inhibition has been demonstrated to potentiate cisplatin-mediated toxicity in several tumour types. We have tested the efficacy of the PARPi AZD2281 (Olaparib) alone or in combination with cisplatin in the treatment of refractory TGCTs.

**Material and Method:** matched sensitive (S) and resistant (R) to cisplatin testicular cancer cell lines (GCT27 and SuSa) were used to perform short and long term viability assays with the aim of evaluating the efficacy of PARPi treatment in monotherapy or in combination with cisplatin in vitro. Homologous recombination (HR)-mediated DNA damage repair efficiency was evaluated after ionizing radiation (IR) or olaparib treatment by counting γ-H2AX and Rad51 foci in both cell lines (S and R). Gene and protein expression levels were quantified by qReal Time (RT)-PCR and Western blot. At a preclinical level, relevance of PARPi treatment was evaluated in orthotopically engrafted primary TGCTs resistant to CDDP.

**Results and Discussion:** we have observed a significant decrease in cell viability in both sensitive and resistant cells after PARPi treatments, either as a single agent or combined with CDDP. Moreover, CDDP-sensitive cells present a delay in HR-dependent DNA damage repair, as shown by slower DNA-damage associated foci clearance, suggesting the presence of defects in this pathway. gRTP-PCR and Western blotting analysis of the main proteins involved in this pathway are currently being analysed. On-going *in vivo* experiments in a orthotopic xenograft nude mouse model with either (i) tumours with acquired resistance to CDDP generated in mice; or (ii) tumours already refractory in the patient, are showing promising results.

**Conclusion:** *In vitro* studies identify PARPi AZD2281 as a potential drug for refractory TGCT treatment. This is supported by our preliminary preclinical results obtained in vivo in a testicular tumour xenograft model.

**Novel Eight-target FISH Approach for Profiling Clonality of High-hyperdiploid Paediatric Acute Lymphoblastic Leukaemia (HHD-pALL)**


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**Background:** The clinically significant high-hyperdiploid (HHD) subgroup of paediatric acute lymphoblastic leukemia (pALL) is featured with a modal chromosomal number of 51–67 and comprises 30% of all pALL cases. Ablate fluorescence in situ hybridization (FISH) is the most reliable technique to investigate such abnormalities, there is no commercially available probe set(s), thus, we have set the aim of developing a multiprobe FISH assay to investigate clonality of HHD pALL.

**Material and Methods:** BAC and plasmid clones carrying sequences for chromosomes X, 4, 6, 10, 14, 17, and 21 were amplified using E. coli. All probes were labeled with both nick translation and random priming (RP) and were tested with four different fluorochromes (Syqua, -green, -gold and -red) for optimization (e.g., probe-fluorochrome pairings). Quality was controlled via both manual and automated analysis; the latter implying automated microscopy. Probes were eventually labeled using RP with an increased amount of template (8 ng/ul) which ensures the yield of labeling significantly. Efficiency was considered sufficient when probe solution exceeded the concentration of 100 ng/ul. ELFO depicts unambiguous proof of appropriate DNA content. A representative FISH signal intensity and signal-to-noise ratio were presented. Following optimization, probes were grouped into work-kits of four; consecutive analysis via automated relocation enabled single cell investigations using all eight probes.

**Results:** Autoantibody repertoire was determined by analysis of more than 12000 cells from 20 healthy individuals which proved to be 98.21, 95.62, 97.12, 94.03, 93.38, 95.64, 97.10 and 98.74 % (average 96.23%) for probes of chromosomes 4, 6, 10, 14, 17, 18, 21 and X respectively. According to preliminary clinical results (18 patients) gains of chromosomes X and 21 were presented at the highest level suggesting their early occurrence during the formation of HHD pattern; while amplification of chromosomes 17 proved to be of the latest event. Comparing paired samples received from the same patient at initial diagnosis and relapse, it has been found that an increased rate of clonal heterogeneity is associated with disease progression.

**Conclusions:** Our unique multiprobe FISH assay appears highly sufficient to investigate aneusomies of HHD pALL patients. Comparing the cell-based results of the clinical trial in progress, to the clinical outcomes, we predict that clonal heterogeneity may have a significant prognostic value.

**Malignant Proliferative Disorder of Leukocytes Preceded by Father–son Psychiatric Traumatisms and Impaired Trustworthiness/Functionality of a Social Relationship ‘father–son’**

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**Background:** The fatherhood crisis, father’s role deficit, and family disintegration are serious phenomena of human society. They can have negative impact on the healthy mental and physical development of an individual, many times even with permanent pathological consequences. Malignant proliferative disorders of leukocytes (MDPL) constitute the most important disorders of white cells. The exact etiology of MDPL is not known. However, a number of factors have been implicated (heredity, environmental factors, infections, associations with disorders of immunity). We are describing acute myeloid leukaemia and Hodgkin’s lymphoma in two young men exposed to psychosocial stress due to the deficit of the man’s role of their father (father–son psychiatric traumatism) in their social relationship ‘father–son’.

**Material and Methods:** Subjective and objective examinations. Formalin-fixed and paraffin-embedded biopsy specimens with bone marrow and lymph node were besides conventional histological staining evaluated histochemically, by a light microscope. In both men we examined trustworthiness/functionality of their social relationship ‘father–son’ and personal features of ‘their father’ by a questionnaire method.

**Results:** Case Reports. 20 year old (Adam) and 21 year old (Boris) men with psychoneurotic syndrome were admitted to the clinic of haematology with the suspecting MDPL. In Adam a preleukaemic syndrome there was present for a few months ago. At admission he had clinical manifestations due to bone marrow failure and due to organ leukemia infiltration. A combination of routine blood picture and bone marrow picture showed acute myeloid leukaemia. In Boris there was in history a low-grade fever with night sweats and weight loss, fatigue, weakness. In clinical features painless, movable and firm lymphadenopathy dominated. Biopsy of the lymph node showed Hodgkin’s lymphoma. Psychiatric-psychological examination revealed sensitive, in men whom trustworthiness/functionality (confidence of a son in) of a social relationship ‘father–son’ is minimal to none. In the long term men since the childhood were exposed to serious psychosocial stress from father–son psychiatric traumatism (deficit of a role of their father: aggressiveness, indifference, absence) and frustration from not fulfilled desire for a functioning social relationship ‘father–son’.

**Conclusions:** Seemingly innocent psychosocial stressors (father–son psychic traumatism) could be possibly associated in susceptible individuals also with MDPL.

**Diagnostic and Prognostic Relevance of Tumour-associated Autoantibody Signatures in Gastric Cancer**

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**Introduction:** Autoantibodies against tumour-associated antigens are very attractive biomarkers for the development of non-invasive serological tests for the prognosis and early detection of cancer. In the current study we explored the autoantibody repertoire in patients with gastric cancer (GC) in order to identify a signature with the diagnostic relevance and examined its correlation with the overall survival and clinicopathological parameters.

**Material and Methods:** We applied T7 phage display-based SEREX technique to identify a representative set of antigens eliciting humoral responses in GC patients and exploited them for the production of phage-displayed antigen
MicroRNA Expression Profiling of Stage I Ovarian Carcinoma Reveals Signatures Characterizing the Different Tumor Grades and Histotypes

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Microarrays. To identify autoantibodies with the highest diagnostic value, a 1150-feature microarray was tested with sera from 100 patients with GC and 100 control sera and then the top ranked 45 antigens were used for the production of focused array that was tested with an independent validation set comprising serum samples from 235 GC patients, 154 peptic ulcer and gastritis patients and 213 healthy controls. After defining cutoffs that discriminate sero-positive and negative signals, the antigens were ranked by the signal intensity and frequency of reactivity and the ‘serum score’ was calculated for each serum by summing up the intensities of the top ranked 45 antigens.

Results: ROC curve analysis showed that the serum score could discriminate GC and healthy controls with AUC of 0.79 (59% sensitivity and 90% specificity), GC and peptic ulcer with AUC of 0.76, and GC and gastritis with AUC of 0.64. Moreover, it could detect early GC with equal sensitivity than advanced GC. The autoantibody production did not correlate with histological type, H. pylori status, grade, localization and size of the primary tumour while it appeared to be associated with the metastatic disease. The total serum score was significantly associated with shorter overall survival (log-rank test p = 0.00045), however further analysis revealed distinct autoantibody signatures with good and poor prognostic significance.

Conclusion: This study resulted in the identification of cancer-associated 45-autoantibody signature that could discriminate between early GC and cancer-free control sera with higher specificity than the currently known serological biomarkers and is significantly associated with shorter overall survival. Furthermore, this study provided some insight into the pathological processes associated with the production of cancer-specific autoantibodies.

Materials and Methods: A panel of basal-like and luminal breast cancer cell lines were treated with various novel PI3K-pathway inhibitors with different mode of action and two antimicrotubule agents (paclitaxel and eribulin) as single agents or in combination, both in anchorage-dependent and -independent cultures. The antiproliferative and proapoptotic activities of the agents were analyzed by flow cytometry.

Results and Discussion: Microtubule-polymerization inhibitors such as eribulin synergized with PI3K inhibitors to induce cell death in basal-like cell lines, both in wild-type and in PI3K-activated models. In the luminal subset synergy between eribulin and PI3K/mTOR-targeting agents was more evident for PIK3CA kinase domain mutant cells than for PIK3CA wildtype and helical domain mutant cell lines. Not only did the combination of PI3K blockade with eribulin show greater synergy to enhance cell death than the combination with paclitaxel, but also the antiproliferative activity was more efficient in both wildtype and PI3K pathway deregulated models.

Conclusions: Blockade of the PI3K/Akt/mTOR pathway synergizes with agents targeting microtubule polymerization in HER2-negative breast cancer cell lines. The precise mechanism by which the combination confers synergy is under investigation.

Investigating MEK and PI3K/mTOR Inhibition as Rational Therapeutic Strategies in a Mouse Model of Colorectal Cancer Mutant for ApC and Kras

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This study evaluates the effect of MEK inhibition and dual PI3K/mTOR inhibition as novel therapeutic strategies in a pre clinical mouse model of colorectal cancer mutant for ApC and Kras. Activation of oncogenic Kras in the context of ApC loss in the murine intestine accelerates tumourigenesis through activation of the MAPK pathway and leads to a significantly reduced lifespan. We aim to investigate the immediate and therapeutic effects on this conditional transgenic mouse model of adenoacarcinoma to determine whether this is a rational therapeutic strategy in the context of the previously mentioned mutations. To evaluate therapeutic inhibition of the MAPK pathway, tumour bearing VillinCreER ApC"KrasLSL/G12D mice were treated with a MEK inhibitor twice daily to a survival end point. Treatment began at 100 days post induction when mice were known to have a tumour burden. Survival analysis revealed MEK inhibition significantly increases longevity of mice as 75% mice are still alive 280 days post induction (median survival of vehicle treated mice was 160 days post induction). To determine the immediate mechanism of action, mice were administered an acute dose of a MEK inhibitor for 4 hours. This resulted in a pro-apoptotic effect and significant inhibition of the MAPK pathway through a reduction in phospho-ERK. However, this also resulted in compensatory activation of the PI3K pathway through significantly increased phospho-AKT and phospho-S6 ribosomal protein. This suggests that inhibition of the PI3K pathway may also be beneficial for tumours with Kras mutations. We subsequently investigated PI3K/mTOR inhibition to determine the potential as a therapeutic strategy for Kras mutant tumours. Acute treatment of 4 hours with a PI3K/mTOR inhibitor also resulted in a pro-apoptotic effect in tumours and a significant inhibition of the PI3K pathway through a reduction in phospho-AKT and phospho-S6 ribosomal protein. Chronic twice daily dosing with a PI3K/mTOR inhibitor also increased longevity of mice to a median survival of 343 days post induction.

In conclusion, inhibition of the MAPK pathway effectively prolongs survival of mice with Kras mutant tumours however, tumours respond immediately to MEK inhibition by compensatory activation of the PI3K pathway. Chronic inhibition of the PI3K/mTOR pathway also effectively increases longevity of mice and therefore, provides the rationale for combined inhibition of the MAPK and PI3K pathways as a rational therapeutic strategy for Kras mutant tumours.
Th1 and Th17 Profiles Induction and Treg Profiles Repression

Associated With Immunological Responses and Long-term Patient Survival on Melanoma Patients Treated With Dendritic Cells Based Immunotherapy

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Background: Melanoma is the most aggressive type of skin cancer that has shown to elicit spontaneous immune responses, both innate and adaptive. T helper lymphocytes (Th) play a key role in the immune protection. Based on the expression of different transcription factors and, ultimately their distinct cytokine profile secretion, many Th helper profiles have been described. Th1 cells are characterized to secrete IFN-γ and are known to effectors of an effective anti-tumor immune response. A relatively new proinflammatory IL-17 secreting CD4+ T cells (Th17) have been described with anti-tumor capabilities. On the other hand, regulatory T cells (Treg) are known to induce and/or maintain the tolerance to self, but they can also repress the induction and/or activation of effector profiles. Dendritic cells (DCs) are professional antigen presenting cells that can activate Th cells specifically, and can influence the proliferation and the differentiation of T cells. We have developed a protocol to generate DCs in 48 hrs (TAPCells), to treat advance melanoma patients. Our therapy showed two distinct groups of patients, differentiated by their tumor-specific delayed type hypersensitivity (DTH) response, correlated with an increased median survival of those in higher DTH responders (DTH+h) when compared to the lower DTH responders (DTH−) 

Materials and Methods: DCs were differentiated from monocytes, loaded with a melanoma cell lines lysate + TNF-α. Serum and PBMC were extracted at each immunization time. Serum levels of cytokines were determined by ELISA and effector and regulatory T cell populations were measured by flow cytomrtry. At the end of the therapy, skin punch biopsies from DTH were performed and effector populations were determined by immunofluorescence. Additionally, in vitro induction of Th1 (CD4+IFN-γ+), Th17 (CD4+IL-17+), TR1 (CD4+IL-10+), Th3 (CD4+TGF-β+) and CD4+Foxp3+ population by TAPCells were determined by flow cytomrtry.

Results: Patients DTH+ have soluble and cellular factors that are involved in these differential responses. We demonstrated that Th1 and Th17 populations are increased on theses patients after our immunotherapy. Conclusions: Our findings indicated that the TAPCells treatment could be inducing effectors profiles while decreasing regulatory profiles in those same patients. Moreover, in vitro experiments show that TAPCells are capable of inducing antitumor profiles in CD4+T cells. Thus different responses evoked by treated melanoma patients could be essential factors for more effective and personalized therapies.

P53 Mutations Affecting DNA-binding Have Prominent Impact on Survival in Chronic Lymphocytic Leukemia

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Introduction: Prevalence of p53 mutations within chronic lymphocytic leukemia (CLL) patients with p53 mutations correlates with shorter survival and more advanced stage of disease. For these reasons, we attempted to identify the frequency of p53 mutations and its correlation to patient survival in CLL patients containing untreated samples.

Materials and Methods: Both alleles of TP53 gene were analysed; deletion 17p− was detected using interface FISH and TP53 mutations were identified by functional analysis in yeast (FASAY) coupled to sequencing of yeast colonies. Results: TP53 gene was altered in 100 of 550 patients. Majority of affected patients manifested biallelic defect (64% mutation/deletion; 4% mutation/uniparental disomy; 7% mutation/mutation).

Mutation: In addition, 21% of patients carried monosomic mutation, whilst only 4% of patients hadletic 17p− deletion. Missense mutations accounted for 78% of all mutations and were all located in the central DNA-binding domain (DBD) of p53. 60% of the missense mutations were localized in the p53 DNA-binding motif (DBM), structurally well-defined parts of the DBD ensuring a proper contact with the target DNA. In survival analyses, only patients with one discrete mutation were considered. The survival was significantly reduced in patients with both missense (P = 0,001) and non-missense p53 mutations (P=0.001). Moreover, patients harboring TP53 mutations in DBMs manifested a clearly shorter median survival (12 months) compared to patients having missense mutations outside DBMs (41 months; P = 0.002) or non-missense alterations (36 months; P = 0.005). Therefore, the p53 DBMs mutations behave clearly differently in CLL patients despite the fact that all mutations led to a basic loss of p53 transcription activity. This most likely reflects the mutated p53 gain-of-function (GOF) effect, which was observed and evidenced by others in several model systems. Patients’ survival was similar in analyses limited to sole mutations and mutations accompanied by 17p−. This suggests that the presence or absence of the second allele may play a milder than expected role in the GOF effect.

Conclusion: CLL patients with p53 mutations in DBMs constitute the most adverse prognostic group of CLL patients. The disease may become a valuable tool to study mutated p53 GOF effect. Supported by CZ.1.05/1.1.00/02.0068, CZ.1.07/2.3.00/20.0045, CZ.1.07/2.4.00/17.0042, MSM0021622430.

Mitotic Arrest Deficiency Protein 2 (MAD2) and Histone Deacetylase 6 (HDAC6) Present a Complex Relationship in Their Regulation and Expression and Subsequent Impact on Chemoresponsiveness

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Introduction: Ovarian cancer patients presenting with advanced stage (III/IV) cancer are treated with carboplatinum in combination with paclitaxel. Despite a significant initial response rate, fewer than 20% of patients become long-term survivors. We have published that low MAD2 expression levels are associated with reduced progression free survival (PFS) in patients with high-grade serous epithelial ovarian cancer (EOC). Moreover, we have demonstrated that MAD2 expression is down-regulated by the microRNA miR-433 (Furlong et al., 2011). Interestingly, miR-433 also down-regulates HDAC6 (Simon et al., 2010), which uniquely deacetylates α-tubulin prior to HDAC6 binding to α-tubulin. In vitro studies have shown that HDAC6 inhibition in combination with paclitaxel treatment enhances chemoresistance in cancer cell death. To date, an interaction between MAD2 and HDAC6 has not been reported.

Experimental design: MAD2 and HDAC6 immunohistochemistry (IHC) and Western blot analyses were performed to investigate the role of HDAC6 and MAD2 in chemoresistance to paclitaxel in high-grade serous EOC.

Results and Discussion: In vitro experiments demonstrated that overexpression of pre-miR-433, which targets MAD2, resulted in down-regulation of HDAC6 in EOC cell lines. High levels of HDAC6 are co-expressed with MAD2 in the paclitaxel resistant UPN251 and OVCAR7 cell lines. While, all 4 paclitaxel resistant EOC cell lines express higher levels of miR-433 than the paclitaxel sensitive A2780 cells, only ova432 and ova433 demonstrated downregulation of MAD2 and HDAC6. Paclitaxel binds to α-tubulin and causes microtubule polymerization in paclitaxel sensitive cells as demonstrated by tubulin acetylation in A2780 cells. However, paclitaxel failed to cause a significant acetylation of α-tubulin and microtubule stabilisation in the resistant UPN251 cells. Therefore resistance in this cell line may be mediated by aberrantly high HDAC6 activity. We have previously shown that MAD2 knockdown cells are resistant to paclitaxel (Furlong F., et al., 2011; Principe M., et al., 2009). We measured HDAC6 protein expression in MAD2 knockdown cells and showed that MAD2 knockdown is associated with concomitant up-regulation of HDAC6. We hypothesise that the up-regulation of HDAC6 by MAD2 knockdown renders cancer cells more resistant to paclitaxel and increases the invasive potential of these cells. On-going experiments will test this hypothesis. Lastly we have observed differential MAD2 and HDAC6 IHC staining intensity in formalin fixed paraffin embedded EOC samples.

In conclusion, we have reported on a novel interaction between MAD2 and HDAC6 which may have important consequences for paclitaxel resistant EOC. Moreover, understanding chemo-responsiveness in ovarian tumours will lead to improved patient management and treatment options for women diagnosed with this disease.

Postmenopausal Early Breast Cancer Patients – the Relevance of Quantitative ER Values and Follow-up Periods

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Introduction: Distant metastases represent major cause of death for breast cancer patients. Recurrence hazard rate in early breast cancer follows bimodal distribution pattern (about 5 years after diagnosis and slow, continual decrease thereafter). Predictive value of estrogen receptor (ER) has been recognized for decades but, due to inconsistent results, it is not fully clarified while its prognostic relevance is...
questionable and still debated. Within relevant time intervals in both natural and clinical course of disease, we analyzed quantitative ER values in order to determine cut-off points, at minimal p value, allowing us to distinguish patients subgroups at different risk for relapse.

**Material and Methods:** Study included 228 postmenopausal breast cancer patients where 50% patients received no adjuvant therapy and 526 patients received adjuvant tamoxifen treatment. ER concentrations were measured in breast cancer tissue extracts by use of classical biochemical method. Within the relevant time intervals (0−2.5, 2.5−5 and 5−12 years), all patients had equal, maximal follow-up. Survival curves for disease-free interval (DFI) were estimated according to method of Kaplan and Meier and compared by log-rank test.

**Results and Discussion:** In most studies, analyses are based on diverse follow-up intervals, having various median follow-up times. In our study, all relapse-free patients were monitored during whole follow-up periods, relevant for occurrence of distant metastases. For defining ER status in breast cancer, not only that different arbitrary values are used as ER cut-off values but analysis of ER quantitative values is neglected due to use of immunohistochemical. Analysis revealed ER concentration of 52 fmol/mg (p = 0.02) as cut point for pN0 patients indicating prognostic relevance of ER only within follow-up period of 5−12 years after diagnosis. Among patients treated with adjuvant tamoxifen, ER showed predictive value within periods of 0−2.5 (cut-off value at 4 fmol/mg, p = 0.002) and 5−12 years after diagnosis (cut-off value at 158 fmol/mg, p = 0.05) that might be important in planning of endocrine therapy.

**Conclusions:** Within different, relevant intervals of natural as well as clinical follow-up, there is no unique cut-off value for ER expression in breast cancer. Within same, relevant intervals of natural and clinical follow-up, cut-off values for ER expression in breast cancer are not equal.

**NFKB2 Intron 8 G/T Polymorphism in NSCLC Patients – a New Risk and Prognostic Factor**

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**Background:** The members of the NFkB family are among the most important transcription factors in cancer. NFKB1 and the classical pathway have become hot topics of detailed research in the last years, although, little is known relating to the possible role of NFKB2 (alternative pathway of NFKB) in carcinogenesis.

**The aim of this study was to define the relation of the NFKB2 single nucleotide polymorphism rs7897947 with non small cell lung carcinoma (NSCLC) and its association with NFKB2 protein expression.**

**Material and Methods:** We used 242 blood specimens and paraffin-embedded tissue specimens from patients with NSCLC. We also used 251 blood specimens from healthy donors. DNA isolation was performed using the Qiagen DNA blood kit (blood specimens) and the QIAamp DNA FFPE Tissue kit (tissue-specimens). Samples were genotyped using real-time PCR. Immunohistochemical analysis for NFKB2 protein was performed on 109 paraffin-embedded tissue specimens from the same NSCLC patients as above.

**Results and Discussion:** Approximately half of the healthy donors (43.8%) were TT homozygotes, 6.8% were GG homozygotes and 49.4% of them were GT heterozygotes. Patients were 51.2%, 7.4% and 36% respectively. The difference in allele frequencies between healthy controls and patients with NSCLC was statistically significant (p = 0.024). More specifically, patients had higher frequency for T allele, while healthy donors had higher frequency for G allele. In addition, rs7897947 was related to the pathological stage of the disease (p = 0.018). Sixty five point eight percent of stage III patients were detected to be TT homozygotes, while 61.5% of stage II patients carried G allele. Two-year survival of the patients was found to be correlated with snp genotype. Two-year survival of the patients was found to be correlated with snp genotype. In addition, rs7897947 was related to the pathological stage of the disease (p = 0.024). More specifically, patients had higher frequency for T allele, while healthy donors had higher frequency for G allele. Two-year survival of the patients was found to be correlated with snp genotype.

**Conclusion:** T allele was found to predispose to NSCLC development. Rs7897947 polymorphism was associated with two-year survival of stage 2 NSCLC patients and pathological stage.

**The Methylation Pattern of Pancreatic Juice Offers Good Diagnostic Yield in the Diagnosis of Pancreatic Carcinomas Diagnostic**

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**Introduction:** Methylation markers have shown promise in the early detection of pancreatic cancer. We have previously shown that methylation status of promoters of HRH2, EN1, SPARC, CDH13 and APC genes may be specific of pancreatic tumor cells. The aim of this study was to assess the clinical usefulness of the panel of markers of candidate genes in combination with KRAS mutation detection in the diagnosis of pancreatic carcinoma.

**Material and Method:** 136 pancreatic juice obtained during the surgery were analyzed. Four groups of patients were included: 85 carcinomas of the exocrine pancreas (PC), 26 amputally of carcinomas (AC), 10 Intrapancreatic Mucinous Neoplasma (IPMN) and 15 Chronic Pancreatitis (CP). Methylation status of promoters was analyzed using Methylation Specific Melting Curve Analysis (MS-MCA) after DNA bisulfite treatment. KRAS mutations were detected by a conventional PCR with Taqman probes analysis with Light Cycler 480.

**Results:** Markers HRH2, APC and CDH13 offered a good yield in the differential diagnosis of PC vs AC and IPMN and PC (Fishertest: p = 7 × 10−4, 1 × 10−4 and 3 × 10−4 respectively). Differences were not big enough to establish a classifier. The prevalence of methylation in PC was as follows: 79% APC, 59% HRH2, 53% CDH13 and 35% SPARC and 33% EN1. Comparing PC, AC and IPMN CDH13 was methylated in only 23% of ampullary tumors (p = 0.047). The prevalence of HRH2 in IPMN was significantly higher (62%) (p = 0.034).

**Conclusion:** Using the methylation status of all assessed genes as a panel with a cut-off value of two or more hypermethylated genes the panel offered a sensitivity of 75% and a specificity of 92% for the diagnosis of PC vs CP. In this setting KRAS codon 12 mutations alone had a sensitivity of 51% and a specificity of 66%. When assessing pancreatic juice, KRAS did not add to the methylation panel useful information to distinguish between PC and CP (sensitivity 81%; specificity 69%).

**Conclusion:** The methylation status of candidate genes shows specific patterns between distinct histopathological groups. The hypermethylation panel offers a diagnostic yield of pancreatic cancer when assessing pancreatic juice.

**The Prognostic Value of Glutathione (GSH) and Glutathione Peroxidase (GPX) in Breast Cancer**

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**Introduction:** The use of prognostic markers for breast cancer is important for routine diagnosis and research, allowing therapeutic strategies to be defined more efficiently. The expression of GSH and GPX in tumor cells has been evaluated as prognosis predictor and responsive to cytotoxic treatments.

**Methods:** The immunorexpression was assessed in 63 women with invasive ductal carcinoma and correlated with clinicopathological parameters.

**Results and Discussion:** High GSH expression was related to negative estrogen receptor tumors and GPX expression was related to negative progesterone receptor tumors and death. Focusing in 37 patients who received adjuvant chemotherapy/radiotherapy (Group I), high expression of GPX was related to death. The 19 patients who received only adjuvant chemotherapy (Group II) showed high expression of GSH in relation to metastasis. High levels of GPX expression were associated with a shorter overall survival. To confirm this suspicion, the expression of precursor genes to GSH (GCLC and GSS) and GPX gene were checked by Real-Time PCR in the cells treated with doxorubicin in vitro. Doxorubicin treatment was unable to eliminate tumor cells without alterations in gene expression of GSS, but led to under expression of the GCLC and GPX genes.

**Conclusion:** Our results suggest that high levels of GPX may be related to the development of resistance to chemotherapy in these tumors, their responses to treatment, and the clinical course of the patients involved.
Introduction: The renal cell carcinoma (RCC) is responsible for approximately 3% of cancers among adults, being the third most common urological cancer in the world, with its incidence rates increasing 2% per year. Currently, there is no standard screening test for RCC, and one third of patients presents metastasis at diagnosis and, over the course of the disease, metastasis develops in another 50% of patients. The mechanisms involved in RCC development and progression remain unclear, and new biomarkers for early detection, follow-up of the disease and prognosis are needed in the routine practice to improve the diagnostic and/or prognostic accuracy. There is increasing evidence that microRNAs (miRNAs) are involved in cancer development and progression. The analysis of miRNAs expression patterns offers an opportunity for the identification of several tumors, since some of these molecules appear to be differentially expressed in normal vs cancer tissues. Due to the high stability of circulating miRNAs in the plasma, they appear to constitute good biomarkers for RCC. Our purpose was to compare the changes in miRNAs circulating expression levels between RCC patients and healthy individuals.

Material and Methods: We examined the expression levels of selected miRNAs (miR-221 and miR-222) in plasma samples of 42 patients with histopathologically confirmed diagnosis of RCC and in 22 healthy individuals without evidence of neoplastic disease. The miRNAs relative quantification was performed using TaqMan Real-time PCR method. The relative expression was determined using the 2^(-ΔΔCt) method, and expression values were normalized to miR-let-7c. The Student’s t-test or Mann-Whitney U test were used, depending on the data distribution, in order to evaluate statistical differences among the different groups of individuals.

Results and Discussion: We observed statistical differences in expression levels of miR-222 between the control group and the patients group. The RCC patients presented higher circulating expression levels of miR-222 than healthy individuals (2^−ΔΔCt = 7.7; P < 0.018). Regarding miR-221, we observed no statistically significant differences in the circulating expression levels (2^−ΔΔCt = 1.3; P = 0.645) between the two groups. We were able to identify the miR-222 as the potential biomarker for RCC, due its significant association for discriminating patients with RCC from healthy individuals. The microRNAs studied have been described as oncogenic. The up-regulation of miR-221 and miR-222 have been associated with the down-regulation of the p27, conferring to the damaged cells the capacity of progress to the S phase of the cell cycle, promoting their proliferation.

Conclusion: Changes in expression levels of miR-221 and miR-222 may promote tumor development by deregulation of cell cycle. Our preliminary results suggest that changes in miR-222 and miR-222 may allow us to distinguish patients with RCC from healthy individuals, however further studies must be performed. Circulating miRNAs can help us recognize specific expression profiles associated with RCC development, which will allow us to identify new molecular biomarkers for RCC, leading to future individualized therapies.

References:

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Contribution: The circulating microRNA-222 in plasma is a potential biomarker for renal cell carcinoma.
patients will be cured after standard-doses of CDDP, although a 15−20% will not achieve a durable complete remission and they finally die. Epithelial ovarian cancer (EOC) are usually sensitive to initial platinum-based chemotherapy regimens. However, even after a complete clinical response, most ovarian carcinomas do recur, developing resistance to platinum therapy after one or more chemotherapy courses.

Aim: The aim of this study was: (i) the generation of tumor models of TGCTs and EOCs that recapitulate human disease; and (ii) latter in vivo development as tumor models of acquired cisplatin resistance.

Materials and Methods: Fresh surgical specimens of human TGCTs and EOCs were obtained after surgical resection at the Hospital Universitari de Bellvitge and orthotopically engrafted in five weeks nude Swiss mice. To generate tumors with acquired resistance to cisplatin mice were treated with increased doses of CDDP by i.v tail injection.

Results: In the last six years, we have generated a XenoBanc of TGCTs and EOCs after the orthotopic implantation of primary human tumors in nude mice: (i) Non-seminomatous (NSE) testicular tumors; 16 TGCTs were perpetuated in mice and all, NSE anatopys were representing (including pure embyronic carcinomas, choriocarcinomas, yolk sac, and immature teratomas, together with mix tumors composed by two or more of these components); (ii) 35 EOCs were perpetuated representing all tumor anatopys (endometrioïd, serous, mucinous and clear cell carcinomas). All of these tumors was histologically characterized, as a basic genetic characterization realized. In addition, to investigate cisplatin resistance in a same genetic background (sensible vs. resistant) we have developed in vivo tumor models (8 TGCTs and 25 EOCs) with acquisition to cisplatin resistance.

Conclusions: Our newly developed tumor models of TGCTs and EOCs may help in the identification of markers associated with acquired resistance to cisplatin, as they may be useful preclinical models to develop newly therapeutic strategies to overcome cisplatin patient resistance.

[555] Assessment of HER2 Status on Disseminated Tumor Cells in Early Stage Breast Cancer Using a Microfluidic Cell Enrichment and Extraction Technique

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Introduction: Evaluation of HER2 status of disseminated tumor cells (DTCs) in bone marrow (BM) provides information that might be useful for developing trials of targeted therapy against DTCs. We report here the discordance in HER2 status in DTCs in early stage breast cancer detected by fluorescence in situ hybridization (FISH) using a microfluidic cell enrichment and extraction platform (OncoCEE®).

Materials and Methods: BM (1-2 ml) were collected from patients with early stage breast cancer (Stage T1 and T2) in acid citrate dextrose solution (BD, Franklin Lakes, NJ) and anti-clumping reagent (OncoCEE® Sure®). Mononuclear cells were recovered using a Percoll density gradient method, incubated with a mixture of 10 primary capture antibodies (Abs), introduced onto 4-channels microchannels, stained with fluorescent anti-cytokeratin (CK) and CD45 Abs and finally processed for FISH using probes specific to centromere 17 and HER2. The ratio of HER2:CEP17 > 2.0 in any CK+/CD45− and CK−/CD45+ was regarded as positive for HER2 gene amplification.

Results: Bone marrow from 68 patients with stage T1N0 (41), T1N1 (8), T2N0 (11), T2N1 (2), T2N2 (1), T2N3 (2) with HER2− primary breast tumors. The discordance of HER2 status was observed in 22% in DTCs.

Conclusion: The cell enrichment and extraction microfluidic device provided a sensitive platform for evaluation of HER2 gene amplification of DTCs. DTCs acquired HER2 gene amplification in 7% of patients with HER2 negative early-stage primary breast tumors. The discordance of HER2 status was observed in 22% in DTCs.

[896] Circulating MicroRNAs Associated With Early Relapse in Early-stage NSCLC

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Background: Lung cancer is world-wide the leading cause of cancer related death. Even in early-stage tumors the outcome is critically determined by metastasis spread: About 30−50% of patients encounter a recurrence after surgery of lung cancer. At the time of diagnosis, those patients might benefit from additional systemic therapies. Therefore, the stratification of early-stage lung cancer with high risk for recurrence will improve therapy management and patient care. microRNAs have been searched in tissue and serum of cancer patients, and represent promising diagnostic and prognostic biomarkers. The aim of the study was to identify microRNAs in serum associated with early relapse in non-small cell lung cancer.

Materials and Methods: Serum samples and RNA extracts were collected from 232 patients including NSCLC disease and control samples. We performed qRT-PCR based microRNA screening (low-density arrays) from a subset of 40 patients with early (median 9 months) or late relapse (median 48 months). Few microRNA candidates were further validated in serum samples of an independent patient cohort (n=114). Additionally, microRNA expression was analyzed in early and late stage NSCLC, and in COPD disease and benign controls. For a subset of NSCLC patients, we compared microRNA abundance between tissue and serum of the same individuals.

Results: The screening experiment revealed ten circulating microRNAs potentially associated with early relapse in NSCLC. One of these microRNAs was upregulated in an independent patient cohort. This microRNA was found to be upregulated in patients with early relapse, especially in patients with NSCLC stage I and II. However, more frequent relapse events were observed in NSCLC stage IIIa. Multivariate analysis using stage information and microRNA expression revealed improved stratification of NSCLC patients with early relapse. Additionally, circulating microRNAs were also influenced by non-malignant disease like COPD. No significant correlation was observed between serum and tissue microRNA abundance of the same individuals.

Conclusions: MicroRNAs may be promising prognostic biomarkers in early stage lung cancer. The combination of biomarker profiles, clinical and epidemiological parameters may improve the diagnosis of severe cancer diseases and therapy assessment.
A Three-gene Expression Signature Model for Risk Stratification of Patients With Neuroblastoma

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Background: Neuroblastoma is an embryonal tumor with contrasting clinical courses. Despite elaborate stratification strategies, precise clinical risk assessment still remains a challenge. The purpose of this study was to develop a PCR-based predictor model to improve clinical risk assessment of neuroblastoma patients.

Methods: The model was developed using real-time PCR gene expression data from 96 samples, and tested on separate expression data sets obtained from real-time PCR and microarray studies comprising 362 patients.

Results: Based on our prior study of differentially expressed genes in favorable and unfavorable neuroblastoma subgroups, we identified three genes, CHD5, RAF1A1B1 and NME1, strongly associated with patient outcome. The expression pattern of these genes was used to develop a PCR-based single score predictor model. The model discriminated patients into two groups with significantly different clinical outcome (Set 1 5-year overall survival [OS]: 0.93 ± 0.03 vs 0.53 ± 0.06, 5-year event free survival [EFS]: 0.85 ± 0.04 vs 0.42 ± 0.06, both P < 0.001; Set 2 OS: 0.97 ± 0.02 vs 0.61 ± 0.01, P = 0.06, EFS: 0.96 ± 0.01 vs 0.56 ± 0.01 and Set 3 OS: 0.96 ± 0.02 vs 0.56 ± 0.06, EFS: 0.96 ± 0.02 vs 0.43 ± 0.05, both P < 0.001). Multivariate analysis showed that the model was an independent marker for survival (P < 0.001, for all). In comparison with accepted risk stratification systems, the model robustly classified patients in the total cohort, and in different clinically relevant risk subgroups.

Conclusion: We propose for the first time in neuroblastoma, a technically simple PCR-based predictor model that could help refine current risk stratification systems.

[099] FoxG1 Confers Resistance to PI3K Inhibitors Through the Repression of FoxO Activity

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Glioblastoma (GBM), WHO grade IV glioma, is the most frequent and aggressive primary brain tumour of the adulthood and its treatment is nowadays dismals.

The PI3K pathway is a key regulator of most cellular processes. Components of the PI3K pathway are frequently mutated in GBM leading to overactivation of the pathway. Several compounds targeting the PI3K pathway are currently being developed and clinically tested. Besides other actions, PI3K inhibitors restore FoxO function by preventing its phosphorylation.

The FoxO subfamily of forkhead transcription factors are tumour suppressors that regulate a wide variety of genes involved in key cellular processes such as glucose metabolism, oxidative stress, cell cycle and apoptosis.

The forkhead transcription factor FoxG1 plays a key role in the regulation of the FoxO family, a key player in the suppression of FOXO activity. When PLX4720, a selective BRAFV600E inhibitor, was used to treat colon tumours, resistance was recorded. In an attempt to discover rational therapeutic combinations to re-sensitize oncogene driven tumours to apoptosis, treatment of two genetically different BRAF(V600E) mutant colon cancer cell lines with PLX4720 conferred complete resistance to cell death. Even though p-MAPK/ ERK kinase (MEK) suppression was achieved, TRAIL, an apoptosis inducing agent, was used synergistically in order to achieve cell death by apoptosis in RKO(BRAF(V600E)/PIK3CAH1047) cells [2]. Pharmacological suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in RKO cells, indicating the presence of PIK3CA(673K) mutation.

Here we have studied the role of FoxG1 in human glioma as an inhibitor of FoxO activity and the implications of this regulation in treatments with anti-PI3K agents.

The immunohistochemical analysis of a tissue microarray of 90 gliomas showed that FoxG1 was expressed in 75% of human gliomas. Furthermore, FoxG1 expression conferred poor prognosis in GBM.

Using co-immunoprecipitation, we found that FoxG1 bound to FoxO. RT-PCR and WB analyses also showed that FoxG1 repressed the FoxO-mediated induction of cytostatic and pro-apoptotic genes in GBM cells. Moreover, PI3K inhibitor NVP-BKM120 was found to induce expression of FoxO targets and cell cycle arrest in GBM cells. FoxG1 was found to prevent the PI3K inhibitor-mediated induction of FoxO targets and cell cycle arrest.

Finally, in an orthotopic mouse model, FoxG1 knock down enhanced the anti-tumoural activity of FoxG1 inhibitor NVP-BKM120 in GBM.

Hence, this study indicates that FoxG1 confers resistance to PI3K inhibitors through the repression of FoxO activity.

[101] Nuclear Variant ErbB3Δ380 mutations Activate Cyclin D1 expression and Cell Proliferation – A Model for Prostate Cancer Progression?

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We have recently described a nuclear variant of the ErbB3 receptor in the nuclei of the H358 lung adenocarcinoma cell line. In vitro, the nuclear ErbB3Δ380 protein functions as a transcriptional activator that binds to the Cyclin D1 promoter to activate Cyclin D1 protein expression and increase cell proliferation.

ErbB3 has been suggested to play a role in prostate cancer progression, but the mechanisms involved remain unclear. Previous studies have demonstrated a link between ErbB3 activation and androgen receptor (AR) activity. Activation of ErbB3 increases AR transcriptional activity and cell proliferation, likely by suppression of the ErbB3-binding protein (Ebp1). Alternatively, AR activation has been shown to suppress ErbB3 expression in androgen dependent prostate cancer cell lines, leading to increase ErbB3 expression in case of androgen withdrawal.

In order to validate our molecular model, we analyzed the expression and cellular localization of ErbB3Δ380, Ebp1 and Cyclin D1 in prostate cancer cell lines as well as normal androgenic prostate tissues. Together, our in vitro and in vivo data suggest that ErbB3Δ380 could be a good indicator of prostate cancer progression from hormone-sensitive to castration-resistant tumors and that the increased proliferation observed in the latter may be in part mediated through activation of Cyclin D1, triggered by nuclear ErbB3.

Sunday 8 – Tuesday 10 July 2012
Poster Session
Experimental / Molecular Therapeutics, Pharmacogenomics

BRAFV600E Inhibitor Resistance Mechanisms – Co-treatment With TRAIL and PI3K Inhibitors can Sensitize to Apoptosis Resistant Colon Cancer Cells

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Introduction: Sporadic colorectal cancer is a major cause of death worldwide. 30–40% of colorectal tumours bear K-RAS and up to 15% a BRAF mutations. Notably, K-RAS and BRAF mutant neoplasms overexpress TRAIL receptors, which may sensitize these tumours to TRAIL induced cell death [1]. In colon cancer appearance of oncogenic alterations in BRAF or KRAS can lead to co-exist with those in PIK3CA and mutated PI3K has been shown to interfere with the successful application of MEK inhibitors.

Materials and Methods: A panel of colorectal cancer cell lines bearing endogenous BRAF mutations, as well as cell lines with either overexpressed or silenced BRAFV600E were employed. Treatment in 2D or 3D cell cultures was with BRAF, PI3K, HSP90 inhibitors and rational combinations with TRAIL.

Results and Discussion: When PLX4720, a selective BRAFV600E inhibitor, was used to treat colon tumours, resistance was recorded. In an attempt to discover rational therapeutic combinations to re-sensitize oncogene driven tumours to apoptosis, treatment of two genetically different BRAF(V600E) mutant colon cancer cell lines with PLX4720 conferred complete resistance to cell death. Even though p-MAPK/ ERK kinase (MEK) suppression was achieved, TRAIL, an apoptosis inducing agent, was used synergistically in order to achieve cell death by apoptosis in RKO(BRAF(V600E)/PIK3CAH1047) cells [2]. Pharmacological suppression of the PI3K pathway further enhances the synergistic effect between TRAIL and PLX4720 in RKO cells, indicating the presence of PIK3CA(673K) mutation.

Conclusion: BRAFV600E bearing colon tumour cells can confer resistance to BRAF inhibitors. Co-treatment with PI3K inhibitors and TRAIL can sensitize these cells to apoptosis, thus providing an opportunity for potential personalised treatment protocols to be further tested in the clinic.

Reference(s)
Exploring the “Warburg Effect” for Cancer Therapy - Targeting Lactate Exporters in Cervical Carcinoma

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Introduction: The Warburg effect, which consists in the up-regulation of the glycolytic metabolism, even in the presence of oxygen, has been described as an important adaptive mechanism to overcome intermittent hypoxia in pre-malignant lesions. In this context, monocarboxylate transporters (MCTs) emerge as important players due to their dual function as lactate exporters and tumour intracellular pH regulators. We have recently described the up-regulation of monocarboxylate transporter 1 (MCT1) along the progression towards invasive cervical carcinoma. Therefore, we aimed to evaluate the role of MCT1 in cervical cancer survival and aggressiveness.

Material and Methods: The metabolic profile of the human cervical cancer cell lines Ca Ski, HeLa, Hela, SiHa, C33 and HeLa/CT and the inhibition of tumor cell biomass (Sulfurohydamine B assay), cell proliferation (BrdU incorporation), glucose consumption and lactate production varied among cell lines, with Ca Ski, HeLa and HeLa/CT being the most glycolytic cell lines and SiHa showing the lowest rates of glucose consumption and lactate production. When exposing cervical cancer cells to CHX, we found a significant decrease in total cell biomass, proliferation, migration and invasion, but little effect on cell death. Further MCT1 silencing studies (shMCT1) were being conducted to validate the role of MCT1 in cervical cancer.

Results and Discussion: Glucose consumption and lactate production varied among cell lines, with Ca Ski, HeLa and HeLa/CT being the most glycolytic cell lines and SiHa showing the lowest rates of glucose consumption and lactate production. When exposing cervical cancer cells to CHX, we found a significant decrease in total cell biomass, proliferation, migration and invasion, but little effect on cell death. Further MCT1 silencing studies (shMCT1) were being conducted to validate the role of MCT1 in cervical cancer.

Conclusions: In this study, we characterised for the first time the effect of MCT1 inhibition in cervical cancer cells’ survival and aggressiveness. The results herein presented point at MCT1 as a promising therapeutic target for cervical cancer therapy.

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Biological Mechanism of Action of Novel -3-(2,5-dihydropyran- 3-yl)-4-methoxy-1H-indole in Human Leukemic Cell Lines

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Background: Bifurcated compounds are valuable moieties used in asymmetric synthesis, organic dyes and medicinal chemistry. The indole and furan skeletons exhibit biological activities. The combination of two or more different heterocyclic motifs in a single molecule would enhance biological activity significantly. In this study, we sought to determine the mode of action of 3-(2,5-dihydropyran-3-yl)-4-methoxy-1H-indole (DMI) by focusing on the processes that mediate its antileukemic activity.

Material and Methods: The anti-proliferative effect of DMI was determined by flow cytometry, MTT assay and Annexin-V positivity, mitochondrial membrane potential and cell cycle progression were determined by flow cytometry. For toxicity testing, 150 mg/kg/day DMI was orally administered to healthy Balb/c mice for 20 consecutive days and body weight was measured each day. For pharmacokinetics, DMI concentrations in rat plasma were determined by HPLC and estimated using zero-moment nonlinear compartmental method. The changes in apoptotic proteins were detected by western blotting. Morphological changes were observed by confocal microscopy. Absorption spectroscopy was used to determine the activity of mitochondrial respiratory chain complexes and caspase activities. Rates of oxygen consumption were measured with a Clark-type oxygen electrode.

Results: DMI exhibited an IC50 of 7.7 μM in human leukemic U937 cells. DMI induced anti-proliferative activity is associated with increased ROS generation which correlates with increased Annexin-V positivity, inhibition of mitochondrial complex activity, decrease in oxygen consumption and altered levels of pro- and anti-apoptotic proteins. NAC, an antioxidant, inhibited DMI induced ROS accumulation and cytotoxicity. This leads to mitochondrial disruption resulting in cytochrome c release and caspase activation. This culminated in cell cycle arrest, and formation of apoptotic bodies. Throughout the period of treatment, there was no mortality and we found no clinical signs of toxicity at the tested dose of DMI. After oral administration to rats, the pharmacokinetic parameters of DMI were as follows: t1/2 6.0 h, Cmax 104.48 μg/ml, half-life 7 h and AUC(0—∞) 1273.8 h·μg/ml.

Conclusions: Our findings suggest that DMI has promising antileukemic activity involving apoptosis and mitochondrial dysfunction coupled with ability of quick action and clearance, meriting further investigation.

Polymer Coating of Salmonella Circumvents the Warburg Effect - Targeting Lactate Exporters-specific Immune Response

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Introduction: Salmonella has been regarded as an excellent antitumor agent. Preexisting immunity against Salmonella may compromise the efficacy of Salmonella for tumor-targeting and antitumor activities. The purpose of this study is to modulate the tumor-targeting efficiency of Salmonella in the preexisting immunity to Salmonella by coating with poly(allylamine hydrochloride) (PAH) (PAH-modified Salmonella).

Material and Method: Herein, we used naive mice and mice immunized with Salmonella to study the role of preexisting immune response in the antitumor activity of PAH-modified Salmonella. These results indicate that encapsulation of Salmonella effectively circumvented the Salmonella-specific immune response.

Yersinia Pseudotuberculosis L-asparaginase – a Promising New Chemotherapeutic Agent

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Background: L-asparaginases from E. coli (EcA) and E. coli (EcA) were used for acute lymphoblastic leukemia treatment for over 30 years. Treatment efficacy is limited by antibody response and by various side-effects. Drawbacks associated with immune response can be circumvented by sequential therapy with serologically unrelated L-asparaginas. Therefore, search of new enzymes with high therapeutic activity and different serological properties is important for biotechnology and clinical oncology.

Material and Method: We have cloned ansB (YPTB1411) gene from Y. pseudotuberculosis O66CJ2 and constructed stable inducible expression system of L-asparaginase from Y. pseudotuberculosis (YpA) in E. coli BL21 (DE3) cells ("Strategene"). For purification of YpA we used Q-Sepharose and DEAE-Toyopearl column chromatography.

We studied the effect of YpA on the proliferation of different three cell types in the MTT-test. Human lung adenocarcinoma A549, human breast adenocarcinoma MDA-MB-231 and MCF7 lines were provided by the ATCC. Index of cytotoxicity (IC50) represented the concentration of YpA required for 50% inhibition of cancer cells viability in vitro.

In the vivo efficacy we evaluated using female DBA/2 mice bearing L5178y lymphoma cells. Different doses of YpA or EcA were administered i.p. in saline solution on days 1-10. Mice surviving for 60 days with a evidence of disease were considered cured. Sixty-day survivors were not included for calculation of mean survival time. Results of experiments were analyzed using log-rank test. A p-value of less than 0.05 was considered significant.

Results: Towards YpA, IC50 for A549 was 2.23 μg/ml, MDA-MB-231 − 10.0 μg/ml, whereas the concentration of YpA that is required for 50% inhibition of MCF7 was more than 50 μg/ml. These results are identical to that for EcA as the reference substance at similar conditions. The L5178y tumor...
cells were sensitive to YpA. Treatment with YpA 1000 IU/kg per day prolonged the lives of all mice and cured 2 of 13 mice. Compared to control group, the animals treated with YpA showed significant increase in mean survival time, almost 36%, from 17.5 ± 1.8 days to 25.4 ± 3.1 days (p < 0.05). In the ECA group, there were 4 cured mice, the mean survival time was 32.2 ± 5.4. The difference between YpA and ECA groups was not statistically significant. **Conclusion:** This study shows significant antitumor activity of YpA and gives promising data on the possible application of YpA in oncology. However, the moderate effect of YpA by the side of ECA does not point to any clear explanation of practical advantages over ECA. It would be of interest to make further immunological comparisons of YpA and ECA as the practical aspect of obtaining antieleukemic enzymes with minimal cross-reactivities for clinical use.

**Material and Methods:** We examined the activity of pyrimidine and purine metabolic enzymes of intrathecal MTX therapy.因为我们

**Background:** We sometimes experience the methotrexate (MTX) resistant meningocarcinomatosis (MC) which is probably caused by pre-multiple anticancer chemotherapy or during the course of intrathecal MTX therapy. Because of this problem, a treatment method for overcoming MTX resistance has been awaited.

**Material and Methods:** We examined the activity of pyrimidine and purine metabolic enzymes of intrathecal MTX therapy.

**Results:** In R-MM46, OPRT showed no apparent changes during the 24 hours and TK showed a transient increase at 2 hours after MTX administration and then gradually decreased, although TK in O-MM46 showed a sharp decrease. PRPP levels in R-MM46 were higher than those of O-MM46, and then gradually decreased, although TK in O-MM46 showed a sharp decrease at 2 hours after MTX administration.

**Conclusion:** There was no significant difference in the MC mouse survival rate using R-MM46 between controls and MTX-mice treated with the same dose of MTX, although a significantly increased survival rate was observed using O-MM46.

**Results:** In R-MM46, OPRT showed a transient increase at 2 hours after MTX administration and then gradually decreased, although TK in O-MM46 showed a sharp decrease. PRPP levels in R-MM46 were higher than those of O-MM46, and the decrease after administration was less in R-MM46 than O-MM46. These findings indicated that the purine salvage pathway was activated in R-MM46. Moreover, OPRT was increased by 200% at one hour after MTX administration in R-MM46, while O-MM46 showed a gradual decrease, although the level of OPRT in R-MM46 was lower than that of O-MM46 pre-MTX administration. Therefore, we examined the synergistic interaction between intrathecal MTX and oral 6-TG by changing the timing of MTX administration in MC mice using R-MM46. Treatment was started 2 days after inoculation of tumor cells and repeated five times every day. The group given MTX and 6-TG simultaneously and that given 6-TG 2 hours after MTX showed significantly increased survival times compared with control (intrathecal PBS). MTX only (12.5 g/0.05 ml) and 6-TG only (0.04 mg/animal). Two groups (6-TG 2 hours after MTX and 6-TG 6 hours after MTX) showed significantly increased survival rates compared with that of the simultaneous MTX and 6-TG group, although the group given 6-TG 12 hours after MTX showed no increase in survival. The groups pre-exposed to MTX (2 and 6 hours) showed the longest survival periods.

**Conclusion:** These findings showed that pretreatment with MTX contributes to 6-TG toxicity and paralleled changes in enzyme activity. Therefore, this therapeutic regimen may be useful in treatment of MC resistant to MTX.

**Introduction:** The effects of sorafenib on liver regeneration have been studied in multiple animal models. For example, sorafenib treatment of liver regeneration in rats was found to increase the proliferation of liver cells and decrease the number of BrdU and MIB-1 positive nuclei. Additionally, sorafenib treatment was found to increase the expression of ERK1/2 and AKT in liver cells.

**Results:** In the sorafenib-treated group, the liver weight increased from 5 days in control to 14 days in rats continuously exposed to sorafenib.

**Discussion:** Sorafenib inhibited liver cell proliferation 48 hours after PH. A compensatory increase of cell proliferation was seen when treatment was prolonged, indicating an adaptation to the drug. The time for restoration of liver weight increased from 5 days in controls to 14 days in rats continuously exposed to sorafenib.

**Conclusion:** Sorafenib inhibited liver cell proliferation 48 hours after PH. A compensatory increase of cell proliferation was seen when treatment was prolonged, indicating an adaptation to the drug. The time for restoration of liver weight increased from 5 days in controls to 14 days in rats continuously exposed to sorafenib.

**Background:** Human adipose tissue mesenchymal stromal cell (hAMSCs) have emerged as cellular vehicles to deliver therapy to solid tumours, due to their ease of isolation and manipulation, and wound/tumor homing capacity. hAMSCs have been successfully used in suicide gene therapy using a variety of activating gene/prodrug systems. In the current study we demonstrate an effective model of glioblastoma therapy based on the use of hAMSCs genetically modified to express HSV-TK and luciferase genes with in vivo monitoring of tumor and therapeutic cells. In addition, an in vivo imaging platform allowed us to observe the behavior of therapeutic hAMSCs, offering insight into the therapeutic mechanism.

**Material and Methods:** Bioluminescence imaging (BLI) of cells expressing different luciferases allows the simultaneous monitoring of different cell populations, cell distribution, proliferation or differentiation. We stably transduced hAMSCs for expression of Renilla luciferase, HSV-TK and red fluorescent protein, generating RLuc-R-TK-hAMSC, and R87MG human malignant glioma cells for expression of Firefly luciferase and green fluorescent protein, generating Pluc-G-U87 cells. SCID mice were stereotactically implanted in the brain first with Pluc-G-U87 followed by RLuc-R-TK-hAMSC. The therapeutic process in mice subjected to GCV treatment was evaluated in vivo and real time by BLI. Differentiation of therapeutic cells to the endothelial lineage was also assessed by labeling the above Renilla luciferase expressing hAMSCs with a second, Firefly luciferase reporter regulated by the CD31 promoter and imaged in vivo after implantation in tumors. The effect of endothelial lineage differentiation of hAMSC on the therapeutic effect was evaluated by shRNA impairing of Notch1, and assessed by BLI monitoring of tumor response.

**Results:** Continuous monitoring of tumor size by BLI showed that hAMSCs/GCV treatment resulted in a significant reduction (99.8% vs. control) of tumor cell number and prolongation of survival time. In addition, the combination of BLI and confocal microscopy analysis of therapeutic cells suggests that efficient tumor eradication results from hAMSCs homing to tumor vessels, where they differentiate to endothelial cell lineage, intensifying their cytotoxic effect by destroying tumor vasculature and negating nutrient supply. Moreover, hAMSC endothelial differentiation inhibition resulted in an inefficient therapeutic effect compared to that of using hAMSC (64% vs 8% respectively).

**Conclusion:** We propose that the tumor killing efficiency of genetically modified hAMSCs results from their association with the tumor vascular system and that these cells should be used as vehicles to deliver localized therapy to glioblastoma surgical borders following tumor resection.

**Introduction:** Temozolomide (TMZ), a second-generation imidazotetrazine alkylating agent against high-grade astrocytomas such as glioblastoma multiforme (GBM), is the leading compound in a new class of anti-tumor drugs.
Altered methylation by O\textsuperscript{2}-methylguanine DNA methyltransferase (MGMT) leads to TMZ-resistant GBM. Thus, inhibition of MGMT activity is necessary for TMZ effectiveness. (Z)-butylidenephthalide (Z-BP) is a compound be isolated from the chloroform extract of Rhizoma Chuanxiong and Angelica sinensis, and able to induce the apoptosis of GBM cells in vitro and in vivo. Besides, on a preliminary assay shows that n-BP was able to methylyating the promoter of MGMT, as well as reducing the expression of MGMT. Therefore it has the potential to increase the sensitivity to TMZ in TMZ-resistant GBM cells and to work in combination with TMZ to treat TMZ-resistant GBM cells. 

Material and Method: In order to increase the sensitivity to TMZ in TMZ-resistant GBM cells, it works in combination with BP and TMZ to treat TMZ-resistant GBM cells. And we use Calcusyn software to evaluate the effect of Z-BP in combination with TMZ on inhibiting proliferating cells. In addition, to test whether Z-BP induced MGMT silencing, we used RT-PCR and western blotting to detect the MGMT expression. To examine to what extent MGMT expression was due to its promoter methylation, Methylation-Specific PCR (MSP) analysis was used. Finally, xenografted mice were used as a model system to study cytotoxicity of Z-BP and TMZ in vivo.

Results and Discussion: Here we show a synergistic cytotoxic effect on the GBM cell line 8901 with a low dose of Z-BP combined with TMZ. Using Calcusyn software to analyze the combination index of Z-BP and TMZ, we observed significant synergism in decreasing MGMT expression. Because GBM resistance to TMZ is due to increased MGMT activity, we next investigated the effect of Z-BP on a TMZ-resistant brain tumor cell line, GBM22-TMZ) and examined changes in MGMT. The combination of 50 μM Z-BP with 1600 μM TMZ yielded a combination index of 0.649 (an index <1 indicates synergism), and 100 μM Z-BP with 1600 μM TMZ yielded a combination index of 0.581, indicating that increasing Z-BP enhanced the synergism. In GBM22-TMZ cells, Z-BP also enhanced MGMT promoter methylation and decreased MGMT protein expression in a dose-dependent manner. Using a GBM22-TMZ xenograft tumor model, Z-BP combined with TMZ showed significant antitumor effects (p < 0.01) compared with a single drug. Finally, a survival study with Kaplan–Meier analysis demonstrated a significant benefit of the Z-BP / TMZ combination compared to TMZ alone (P < 0.05).

Conclusion: In summary, the combination of Z-BP and TMZ shows potential clinical relevance for treating TMZ-resistant GBM.

Effect of Gemcitabine on the Growth of Intracranial Tumors in Mice

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Background: Now there is a little information about the ability of gemcitabine to penetrate haematopoetic barrier and to provide a therapeutic effect on brain tumors. Therefore we studied influence of gemcitabine on the Ehrlich tumor and lymphosarcoma LIO-1 were transplanted intracranially in mice. 

Materials and Methods: Ehrlich tumor and lymphosarcoma LIO-1 were transplanted intracranially in 79 mice in control group and TMZ group. For the control group, it was injected intraperitoneally (i.p.) at a single dose of 25 mg/kg or 96 hours after intracranial tumor transplantation of the tumors. Influence of gemcitabine on life span of mice was estimated. 

Results and Discussion: In mice inoculated intracranially with Ehrlich tumor single i.p. injection of gemcitabine at a dose of 25 mg/kg 24 hours after inoculation increased the mean life span up to 19.3±0.9 days, compared with one of 11.7±4.0 days in the control group. Therefore, increase of life span (ILS) was 54% (p = 00000023). For 14 days after the tumor transplantation in control group of 12 animals the all 12 (100%) mice have died, while in gemcitabine group of 10 mice only 3 (30%) mice have died (p = 0.0003). A single i.p. injection of gemcitabine at a dose of 25 mg/kg 96 hours after intracranial transplantation of lymphosarcoma LIO-1 increased the mean life span up to 13.5±0.5 days, compared with one of 10.9±0.4 days in the control group. Therefore, ILS was 24% (p = 0.0003). For 14 days after the tumor transplantation in control group of 18 animals the all 18 (100%) mice have died, while in gemcitabine group of 13 mice only 6 (46.2%) mice have died (p < 0.0001).

Conclusion: Gemcitabine has a direct therapeutic effect on the brain tumors in mice were transplanted intracranially with Ehrlich tumor and lymphosarcoma LIO-1.
CII ubiquinone (UbQ)-binding site (Q$_0$). Mutation of the UbQ-binding site S68 within the Q$_0$ of the CII's SDHC subunit suppressed both ROS generation and apoptosis induction in response to MitoVES. In contrast, MitoVES also significantly suppresses tumour progress and the associated angiogenesis.

**Conclusions:** We propose that mitochondrial targeting of VES maximises its anti-cancer efficacy, endowing it with a substantial translational relevance.

### [916] Modeling Pharmacological Inhibition of Bruton's Tyrosine Kinase (BTK) as a Therapy for Insulinoma and Pancreatic Ductal Adenocarcinoma


**Results:** We observed intercellular transfer of Pgp from donor cells to recipient cells which after co-culture became Pgp$^+$; also, clusters of Pgp were identified in recipient cells suggesting the presence of MPs. Recipient cells showed an enhancement of Pgp mRNA expression, and following treatment with drugs cells showed a decreased of apoptosis index. These cells showed per or nuclear YB-1 staining which was observed in all the other drugs. Also, it was observed an enhancement of EGFR expression and its peri and nuclear localization, indicating its activation. The negative regulator of NFkB, IκBα, was degraded; and thus NFkB was translocated to the nucleus. Parallel to this, it was observed an enhancement of survivin and XIAP mRNA and protein expression. The miR-21, -27a and -451 were upregulated, supporting the MDR acquisition in recipient cells. No changes were observed in Pgp, XIAP, survivin, YB-1 and IκBα in recipient cells cultured with conditioned medium. In MPs analysis was identified for the first time survivin, XIAP, YB-1 and miR-21 expression, along with Pgp, miR-27a and -451.

**Conclusions:** Sensitive cells acquired non-genetic Pgp and IAPs expression, and consequently, MDR phenotype through resistant cells-derived MPs. Also, YB-1 and NFkB pathways could be involved with upregulation of mRNA Pgp and IAPs, and additionally contribute to activation of EGFR. The presence of miRNAs supports the recipient cells MDR phenotype. These findings contribute to our knowledge for the emergence of MDR in cancer cells and could be helpful for new treatment approaches.

### [920] The Effects of N-Acetyl-L-Cysteine on Bleomycin Induced Apoptosis Were Determined in Malignant Testicular Germ Cell Tumours by Flow Cytometry

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**Background:** Bleomycin is used commonly in the treatment of testicular cancer. Bleomycin causes an increase of ROS resulting in oxidative stress and induces apoptosis. Oxidative stress has been shown to induce apoptosis in cancer cells. Therefore, one might suspect that antioxidants may inhibit reactive oxygen species (ROS) and prevent apoptosis of cancer cells. There is an intense argument on the concurrent use of antioxidants with the conventional cancer treatments. This argument was based on the fact that some chemotherapy drugs generate reactive oxygen species (ROS) and antioxidants may prevent cancer cells to be killed by ROS. N-Acetyl-L-Cysteine (NAC), a compound known to have powerful antioxidant properties, was the property of the study, in our study we examined the effects of NAC on oxidative stress created by Bleomycin.

The aim of our study was to clarify the molecular mechanism of apoptosis which induced by Bleomycin and the effect of apoptosis in human testicular cancer cell line. We have chosen the wild-type p53 expressing cell line, NTERa-2 (NT 2).

**Material and Method:** Apoptosis was detected using an FITC Annexin-V Apoptosis Detection Kit by using flow cytometry. Control, Bleomycin, NAC and Bleomycin+NAC groups were examined. We incubated NT 2 cells with different concentrations of Bleomycin (400 mg/ml) and NAC (5 mM) for 6 hours.

**Results and Discussion:** NT 2 cells were incubated with Bleomycin, NAC and Bleomycin+NAC for 6 h (pre-apoptotic time). The percentage of apoptosis was determined by FACS analysis utilizing Annexin V and PI. Incubation of the cells with 400 μg/ml Bleomycin induced apoptosis 48.6% (Q1-pre-apoptotic side and Q2-2 post-apoptotic side) detected compared to the control cells. Incubation with 5 mM NAC did not increase significantly percent of apoptosis compared to the control cells. Pretreatment of the NT 2 cells 400 μg/ml Bleomycin with NAC at 5 mM drastically reduced the proportion of apoptotic cell populations from 48.6% to 29.5% (Q1-1 and Q2-2) compared to Bleomycin group.

Our results indicated that Bleomycin mediated apoptosis was suppressed by NAC in NT 2 cells.

**Conclusion:** Finally, we think it is important for prognosis of the disease of the chemotherapeutic treatment of patients with testicular cancer all the experiment results. During treatment Bleomycin kills testicular cancer cells by generated ROS. Because of antioxidants prevent ROS, we believe that the use of antioxidants during treatment with Bleomycin negatively affect the treatment process.

### [921] Furin-based Saccharide Mimetics Inhibit Tumour Cell Adhesion, Migration and Distinct Stages of Angiogenesis


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**Introduction:** Tumour cell adhesion to extracellular matrix proteins (ECM) is mediated by protein-protein and protein (lectin)-oligosaccharide interactions. Angiogenesis, which involves stages of endothelial cell adhesion and migration, is vital for tumour growth and metastasis formation. Since sugar molecules are involved in both cellular activities, we further developed furan-based saccharide mimetics, which were previously shown to interfere with these cell-ECM interactions (Kim EV. et al. ChemBiochem 2005, 6, 422).
**Methods:** The lead compound 3-[1-D-galactopyranosyloxy]-4-sulfato-methyl-furan (GSF) was modified by adding a second sulphate to the 6 position of galactose. Galactose was substituted with glucose (Glusf) and the sulphate in furan with fluorine (GFF). Frank M. et al. Europen Patent Appl. 11000103.9, 2011. The human melanoma WM-266-4 and HMBEC-60 cells were seeded. EB was added with the sulfonfuran-blue B (GSSF). A two-dimensional migration of the melanoma cells was determined with the ‘wound-healing assay’, as well as the OrisTM assay. Adhesion of cells to the ECM proteins was determined by labelling adherent cells with methylene blue.

**Results and Discussion:** Cytotoxicity of Glusf and GSF were comparable with 5 mM being cytostatic after more than 48h. GSSF was more toxic, here 2.5 mM was cytostatic and 5 mM cytotoxic. After 24 h none of the compounds damaged cells up to 5 mM. At this concentration, GSF effectively inhibited wound healing of WM-266-4 cells. GFF was active at 1 mM and GSSF at 0.1 mM. The quantitative effects determined with the OrisTM assay, were similar if less pronounced. Cell adhesion of melanoma cells to fibronectin was more effectively inhibited by Glusf and GFF than by GSF. Adhesion of HMBEC-60 cells to ECM proteins was not inhibited to a greater extent by GSSF than by GSF. Earlier studies had led us to postulate that furan-based saccharide mimetics bind to integrins rather than lectins and blind docking/molecular dynamics studies showed GSF (Mariano G. et al submitted) and its derivatives to bind to the RGD-binding site on integrin vβ3.

**Conclusions:** The optimisation of our lead compound GSF was successful and first structure-activity relationships can be deduced: melanoma and endothelial cells react differently to the new compounds, although, they had shown similar sensitivity to GSF. Optimisation was mainly achieved for melanoma cells’ migration and adhesion. Both properties are important for the metastatic spread of solid tumours. Chelating properties in a molecule, achieved with a second sulphate improved anti-migratory property 50-fold, concomitant with only a mild increase in cytotoxicity. Replacement of galactose by glucose improved the anti-adhesive effect, similar to the substitution of sulphate by fluorine. Molecular modelling studies had predicted the improved activities seen in the cell experiments.

**[922] DRAGO, a P53/p73 Regulated Gene That Modulates Survival in Tumour Prone Mouse Model**

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**Introduction:** DRAGO (Drug Activated Gene Overexpressed) was cloned in our Laboratory and in vitro experiments demonstrated that it is a p53/p73 responsive gene, whose expression is up-regulated in response to chemotherapeutic compounds with different mechanism of action. DRAGO sequence matches KIAA0247, an uncharacterized, highly evolutionary-conserved gene and both transiently and stably transfected cell lines expressing the gene showed toxic phenotype. These evidences are in accordance with a potential oncosuppressive role for DRAGO. Nevertheless in vivo experiments showed that mice knockout for the gene do not exhibit any evident phenotype and their lifespan is comparable to wild type mice.

Our hypothesis is that DRAGO may cooperate with p53 in counteracting tumor cells. To verify this hypothesis we crossed p53 knockout and DRAGO knockout mice.

We assessed genotype distribution in the offspring and compared survival of double transgenic mice with single p53 knockout mice, which develop an evident phenotype and their lifespan is comparable to wildtype mice. On a p53+/− background only DRAGO+/+ mice displayed prolonged survival compared with DRAGO−/− and DRAGO+/− mice (570 days) compared with DRAGO−/− and DRAGO+/− mice (446 and 431 days respectively). DRAGO genotype did not influence tumor spectrum as all genotypes developed sarcomas (30%), while p53+/− subpopulations developed most sarcomas (57%) and secondarily lymphomas (20%) and carcinomas (20%).

**Conclusions:** Cross p53 and DRAGO knockout mice allowed us to demonstrate the oncosuppressive role of DRAGO in vivo and its cooperation with p53. Moreover, both DRAGO alleles determined a positive effect in survival decrease for p53−/− mice, while on a p53+/− background deletion of one or both DRAGO alleles reduced survival to the same extent. Furthermore DRAGO genotype did not affect tumor incidence since p53−/− and p53+/− subpopulations displayed tumor distributions in accordance with the literature.

**[923] Characterization of the Activity of Human Immunodeficiency Virus Protease Inhibitors Against Acute Myeloid Leukemia in Vitro**

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**Introduction:** HIV protease inhibitors (HIV-PI) are oral drugs for HIV treatment with putative antitumor activity via the induction of ER stress and the inhibition of p-AKT and proteasome activity. Several clinical trials explore HIV-PI in solid tumors. We here characterize the activity of HIV-PI on AML cells to provide a basis for future clinical testing in AML.

**Material and Method:** We evaluated the effects of HIV-PI AML cells regarding cytotoxicity, proteasome activity, ER-stress induction and AKT-phosphorylation, and further evaluated synergistic activity with proteasome inhibitors, lenalidomide and sorafenib in vitro (AML cell lines, AML patient blasts).

**Results and Discussion:** Lopinavir, Nelfinavir, Ritonavir and Saquinavir showed biological and molecular activity at concentrations within or near therapeutic drug levels (10−20 μM). In this dose range, they triggered ER stress-induced apoptosis, inhibited AKT-phosphorylation and showed synergistic cytotoxicity with bortezomib, carfilzomib, lenalidomide and sorafenib. Cell lines and normal control cells were significantly less sensitive towards HIV-PI induced cytotoxicity than primary cells. Nelfinavir was the only HIV-PI with proteasome-inhibiting activity in the tested dose range in intact cells, inhibiting also the bortezomb- and carfilzomib-insensitive i2 proteasome subunit.

**Conclusion:** Nelfinavir, ritonavir, lopinavir and saquinavir in the 10−20 μM dose range are cytotoxic against primary AML cells in vitro, in contrast to control cells or cell lines, and act synergistically with bortezomib, carfilzomib, lenalidomide and sorafenib. Only nelfinavir inhibits proteasome activity in intact cells in vitro and in vivo. We consider nelfinavir as the most promising candidate HIV-PI that warrants clinical tested in AML.
core targeting topoisomerase II with a spermine moiety facilitated F14512 selective uptake into cancer cells. In this study, we report the in vivo antitumoral activity of F14512 against human AML models, established from patient samples.

**Methods:** AML cells, collected from 3 different patients, were established onto NSG mice (LAM-2, LAM-7 and LAM-8). These AML samples exhibited a normal karyotype, with FLT3-ITD, NPM1 and DNMT3A mutations which proved stable over serial transplantations in vivo.

**Results:** After multiple i.v. administrations of F14512, 3 times a week for 3 weeks, an extensive reduction of AML cell number (98–99%) was observed in LAM-2 and LAM-7-bearing mice. This antitumoral activity was recorded on the basis of flow cytometry, q-PCR and histology assessments. The antitumor effects of F14512 on LAM-18 bearing mice were less pronounced with an inhibition of AML cell growth of 42%. Mechanisms of F14512-induced cell death in vivo in these AML cells are currently investigated, and preliminary data suggest that senescence is involved. We also show in vitro and in vivo synergistic effects of F14512 in combination with AraC, one of the frontline chemotherapeutic agents for AML. These results were obtained using the HL-60 cell line and the LAM-2 patient AML model.

**Conclusions:** Collectively, these results demonstrate that F14512 exhibits a marked in vivo antitumoral activity, supporting its clinical development. Phase I clinical trials in oncology are ongoing with this novel promising drug candidate.

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**[928] KRas Mutation Analysis by PCR – Comparison of Two Methods**

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**Introduction:** KRAS mutation analysis is a companion diagnostic for the use anti-EGFR antibodies in colorectal cancer, and there is evidence to suggest that detection of BRAF mutation is also important in these patients. The methods used to establish KRAS mutation are essentially divided into sequencing and PCR based methods. While sequencing can lack sensitivity, particularly in the presence of large amounts of wild-type DNA from infiltrating cells, it does have the ability to find many more mutations than most commercially available PCR methods. In contrast, PCR methods often have better sensitivity, but poorer coverage of all possible gene mutations. Direct comparison of newer diagnostic methods with existing methods is an important part of validation of any new technique. In this study, we have compared the TheraScreen® (Qiagen) assay with the new TaqMan® Mutation Detection assays (TMDA) based on Competitive Allele Specific TaqMan PCR technology (castPCR™ Life Technologies) to determine equivalence for KRAS mutation analysis.

**Methods:** DNA was extracted by Maxwell (Promega) magnetic beads from two punch biopsies obtained from areas of colorectal cancer identified by a pathologist in blocks of formalin-fixed paraffin-embedded tissue in 93 cases. The ARMS-based TheraScreen® assay was performed according to the manufacturer’s instructions, as was the TaqMan® Mutation Detection assays. All assays were performed on an Applied Biosystems 7500 Fast Dx real-time PCR machine (Life Technologies). The data were collected and discrepant results retested with newly extracted DNA from the same blocks in both assay types.

**Results:** Of the 93 cases included, 47 were wild-type (WT) for KRAS, and 33 had KRAS mutations. The initial runs identified just three cases with different results between the two assay types, with complete concordance in 90/93 cases. One sample was negative in Therascreen and borderline positive (dCt = 9.41) by TaqMan® Mutation Detection assays, and was retested as WT in both assays. One sample was WT on retesting by both Therascreen and TaqMan® Mutation Detection assays, but had been mutant by the first Therascreen. The third sample was mutant in Therascreen and borderline positive WT with TaqMan® Mutation Detection assays, but on retesting mutant in both assays. Ten cases showed BRAF mutation (V600E is not included in Therascreen) and in one of these there was also a KRAS mutation. TaqMan® Mutation Detection assays Ct values were on average 0.8 cycles lower than Therascreen, suggesting marginally greater sensitivity.

**Conclusion:** There was excellent correlation between the two methods, though TaqMan® Mutation Detection assays include BRAF and shows slightly better sensitivity than Therascreen, though this is unlikely to be clinically significant.

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**[929] MiR-375 Regulates Cell Proliferation and Cell Cycle Arrest Through the Downregulation of RON in Human Gastric Cancer Cells**

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**Introduction:** The receptor d’oréin nantais (RON), receptor tyrosine kinase is associated with cell proliferation and tumorigenesis in epithelial cancer cells. The expression of RON plays an important role of the development and metastasis in human gastric cancer, but its underlying mechanisms of action are not clear. MicroRNAs (miRNA) are known to play essential regulatory roles in many cellular processes. Many of miRNA targeted mRNAs encode gene essential for the cell proliferation, survival, apoptosis, and invasion during tumor progression.

**Material and Methods:** We confirmed direct binding to miR-375 and 3’UTR of RON use bioinformatics and the expression of miR-375 in gastric cancer cell lines and gastric cancer tissues was confirmed by qR-PCR. Luciferase reporter assay and western blot were used to examine the RON target gene.

**Results and Discussion:** The RON expression was controlled at the levels of both miRNA and protein by miR-375 via direct targeting of the 3’UTR of RON. Mir-375 could suppress cell proliferation and cell cycle arrest, at least partially, via the downregulation of RON expression.

**Conclusion:** This study suggests that expression of miR-375 and RON are inversely related and miR-375 might act as a potential therapeutic target in human gastric cancer cell.

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**[933] Antiprofiletive (E)-4-aryl-4-oxo-2-butenoic Acid Phenylamides, the Chalcone-aryloxyacetic Acid Chimera**

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**Background:** Chalcones exert diverse biological activities: antioxidant, anti- inflammatory, antiangiogenic, and anticancer. Also, phenylcinnamides covalently bind to critical Cys residues of tubulin, causing mitotic arrest and finally apoptotic cell death. Furthermore, another type of molecules comprising ketovinyl moiety, i.e. aryloxyacids, exert antiproliferative activity toward malignant cell lines. Following this rationale, we have designed chalcone-aryloxyacetic acid chimera (title compounds), by incorporating amidic moiety between ω,ω-unsaturated carbonyl moiety and B ring of chalcone. The aim of this work was to assess the effects of seventeen newly synthesized (E)-4-aryl-4-oxo-2-butenoic acid phenylamides on survival and proliferation rate of three different malignant cell lines, as well as on the normal human peripheral blood mononuclear cells (PBMC) in vitro.

**Material and Method:** In vitro cytotoxicity of the compounds was tested using MTT assay against human cervix adenocarcinoma (HeLa), human melanoma (FemX), and erythromyeloblastoid leukemia (K562) cells. Some of the compounds were also tested against healthy PBMC, that were unstimulated, or stimulated for the proliferation with phyllohegamulgin. The compounds were incubated with target cells for 72 h, then their antiproliferative potency (IC50 values) and cell survival were determined. The mechanism of action was examined by flow cytometry using propidium iodide to label DNA. After treatment of HeLa cells with IC50 of the compounds, the cells were harvested and analyzed.

**Results and Discussion:** Tested (E)-4-aryl-4-oxo-2-butenoic acid phenylamides have significant cytotoxic effects toward malignant cell lines, with IC50 varying from 0.68 to 2.92 μM on HeLa cells, 0.34 to 1.26 μM on FemX cells and 0.34 to 0.62 μM on K562 cells, as obtained by MTT assay. Equally important, cytotoxicity against PBMC is lower, with IC50 ranging from 1.15 to 2.27 μM for unstimulated and from 1.37 to 2.83 μM for cells stimulated with phyllohegamulin. Cell cycle analysis of HeLa cells treated with some compounds, which led to accumulation of more cells in S phase and a reduction of cell population in G2/M phase, suggests that examined compounds probably inhibit DNA synthesis of the malignant cells.

**Conclusion:** Results obtained in this study indicate that tested compounds have excellent antiproliferative activity and satisfactory selectivity, and can be considered as the leads for the further investigations.

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**[934] Nk-lysin Derived Peptide (NK-2) Sensitizes Resistant Cancer Cells to Classic Chemotherapeutics by Selective Killing of P-glycoprotein Over-expressing Cells**

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**Background:** Natural antimicrobial host defense peptides are membrane-active agents. These peptides are attracted by negatively charged cancerous plasma membranes. NK-2 represents a highly conserved region of Nk-lysin, an alpha-helical antibacterial effector protein from porcine NK- and T-cells. P-glycoprotein (P-gp), a transport pump, is frequently over-expressed on membrane surface of multi-drug resistant (MDR) cells. P-gp actively extrudes chemotherapy drugs from cancer cells and highly contributes to MDR phenotype. As P-gp expression could affect lipid packing in membrane bilayer and change membrane polarization, which is essential for peptides’ action, we assumed that NK-2 could specifically kill MDR cells.
Material and Methods: We employed sensitive human cancer cell lines (NCI-H460, DLD1) and their corresponding MDR cell lines (NCI-H460/R, DLD1/TxR) with high expression and inhibitory effects of single NK-2 and combined treatment with doxorubicin/paclitaxel were assessed by MTT test. The effect of P-gp inhibitors (tariquidar and verapamil) on NK-2 activity was determined bytrypan blue staining. The mRNA expression of mdr1, which codes for P-gp was analyzed by RT-PCR. Flow-cytometric analyses were used to determine the accumulation of P-gp substrate (rhodamine 123) and to quantify the level of P-gp expression.

Results: The effective inhibitory concentration of NK-2 (IC50) did not significantly differ between resistant and sensitive cells. However, we showed that NK-2 more readily eliminates P-gp positive cells than P-gp negative cells. Also, NK-2 was able to increase the accumulation of P-gp substrate (rhodamine 123). The results obtained with P-gp inhibitors recognized that P-gp activity is important for NK-2 induced cell death. Specifically, the inhibition of P-gp activity by tariquidar and verapamil diminished the effect of NK-2. The expression of mdr1 is significantly decreased in resistant cells pretreated with NK-2. Additionally, NK-2 sensitized MDR cells to doxorubicin and paclitaxel treatment by reverting their inhibitory concentrations to the range close to that effective in sensitive cells.

Conclusions: We showed that NK-2 discriminates P-gp positive from P-gp negative cells. In contrast to other MDR modulators that inhibit the P-gp activity, NK-2 could sensitize MDR cancers by selective killing of the P-gp over-expressing cell population.

935 The Interplay Between Telomerase and Epstein Barr Virus (EBV) – Silencing of hTERT untreated EBV Lycyclic

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Background: The lycyclic of EBV promotes the death of infected cells. Therapeutic strategies that exploit the induction of viral lycital replication might, therefore, constitute a useful adjunct to current treatment regimens for EBV-driven malignancies. Previous findings have indicated that high levels of hTERT, the rate-limiting component of the telomerase complex, confer resistance to viral lycical cyclic. In this work, we tested how silencing of hTERT may influence the EBV lycyclic cycle and impacts cell survival and proliferation.

Material and Methods: and hTERT silencing was obtained by infecting EBV-negative (BL41) and EBV-positive (BL41/89B) Burkitt Lymphoma (BL) cell lines and EBV-immortalized lymphoblastoid cell lines (LCIs) with retroviral vectors expressing short hairpin(sh)RNA targeting hTERT (shTERT3.4). Cells, infected with shTERT3.4 or control vectors and cultured with or without the produg ganciclovir, were analyzed for EBV lytic replication, cell cycle and apoptosis.

Results: hTERT inhibition resulted in the induction of BZLF1 and EA-D lytic proteins and EBV replication with release of virions into culture supernatants. hTERT silencing was associated with down-regulation of BART, a negative regulator of AP-1-dependent BZLF1 transcription. Moreover, silencing of hTERT resulted in anti-proliferative and pro-apoptotic effects. In particular, hTERT inhibition induced an accumulation of cells in the S phase, an effect likely due to the dephosphorylation of 4EBP1, an Akt-dependent substrate, which result in a decreased availability of proteins needed for cell cycle progression. Moreover, besides inducing cell death through activation of complete EBV lycical replication, hTERT inhibition also triggered Akt/Foxo3A/Nova-dependent apoptosis in both EBV-positive and EBV-negative BL cells. Finally, ganciclovir enhanced the apoptotic effect induced by hTERT inhibition in EBV-positive BLs and LCIs.

Conclusions: This study demonstrates that combination of antiviral drugs with strategies able to inhibit hTERT expression might result in therapeutic relevant effects in patients with EBV-related malignancies.

937 Inhibitors of Bcl-3 as a Novel Therapeutic Strategy for HER2+ Breast Cancer

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Background: BCL-3 (Bcl-3) is a proto-oncogene modulating the nuclear factor kappab (NF-kappab) signaling pathway. The level of Bcl-3 was found to be significantly elevated in breast cancer compared to the normal adjacent tissue and interestingly activated NF-kappab signaling in breast cancer tumors compared to normal breast. Previously we have shown that Bcl-3 deficient mice developed HER2+ tumors significantly later than controls and that Bcl-3 deletion significantly reduced the occurrence and size of lung metastases. Based on these findings we suggest that inhibiting Bcl-3 may be beneficial in metastatic HER2+ breast cancers, mainly in the progression to more aggressive forms.

Materials and Methods: HEK 293 and MDA-MB 231 cell lines were transfected with Bcl-3 WT Flag construct, and Bcl-3 binding mutant Flag construct generated by mutagenesis within the second ankyrin repeat of Bcl-3. We used Alanin Chariot, Interenary Cluster for Applied Genoproteomics, Liege). ELISA anti-Flag coated plate were purchased from Sigma. NFkappab activity was assessed by Luciferase assay system using pG3 reporter vectors from Promega.

All molecular modeling studies were performed on a MacPro dual 2.66GHz Xeon running Ubuntu 9 using Molecular operating Environment (MOE) 2009. All protein structures were downloaded from the PDB data bank (http://www.pdb.org). Bcl-3 structure − PDB code 1KA1, p50 structure − PDB code 1SVC and Ibx p65/p50 structure − PDB code 1NF1

Discussion and Results: Bcl-3 modulates transcription through binding with the NFkappab proteins p50 and p52. Here we report that loss of their interaction prevents Bcl-3 mediated regulation of the NF-kappab signaling pathway and decreases cell proliferation and migration capacity. Using molecular modeling we have constructed a model of Bcl-3-p50 complex by superimposing the ankyrin repeat of Bcl-3 and DNA-bound p50 homodimer onto the structure of the ikappabα-p50/p50 complex. We identified a novel protein-protein interaction domain for p50 within unique seventh ankyrin repeat of Bcl-3 molecule and performed initial virtual screening. These potential small molecule inhibitors were evaluated using ELISA assay detecting the loss of Bcl-3-p50 binding, and by NF-kappab activity assay detecting Bcl-3 over-expressing HEK 293 and MDA-MB 231 cell lines. Two lead compounds were selected for further testing by migration assay in MDA-MB 231 cell line and for chemical optimization.

Conclusion: Inhibition of NFkappab signaling pathway by disrupting Bcl-3-p50 binding has emerged as a novel target in HER2+ breast cancer, while other application remains to be proven. Here we show the identification of novel inhibitors by virtual screening and the evaluation by ELISA binding assay and by NFkappab activity assay.

938 TBr4 and BBr2 as Sensitizers for Photodynamic Therapy Against Human Colorectal Adenocarcinoma – in Vitro Studies

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Background: Photodynamic therapy is capable of inducing cell death by oxidative stress through activation of a sensitizer by light. Porphyrins proved to be photosensitizers (PS) with appropriate characteristics. Recent investigations showed that substitution with hydroxyl groups and the heavy atom effect contribute to enhance the photodynamic activity. The aim of our work was to compare the photodynamic activity of 5,10,15,20-tetrakis(2-bromo-3-hydroxyphenyl)porphyrin (TBr4) and 5,15-bis(2-bromo-3-hydroxyphenyl)porphyrin (BBr2) as PS against human colorectal adenocarcinoma.

Material and Methods: The molecules were synthesized as described. The human colorectal adenocarcinoma cell line WiDr was incubated with the PS, Hoechst and Mitotracker Green FM to perform fluorescence microscopy. Uptake was evaluated by fluorescence spectroscopy. To access photodynamic effect of TBr4 and BBr2 cells were incubated with the PS (50 μM) Internalization is considerably higher than 10 μM). Cells were irradiated until 10J and cell proliferation was analysed after 24, 48 and 72 hours by MTT assay. Dose-response curves were established and the half maximal inhibitory concentration (IC50) was calculated. To verify intrinsic toxicity of the PS the same protocol was performed omitting the irradiation step. To access the cell death pathways induced, cultures treated with 1μM of each PS were submitted to annexin V and propidium iodide and analyzed in a BD FACScalibur™ flow cytometer.

Results: Fluorescence microscopy studies indicated that the subcellular localization of the PS may be the mitochondria, and cellular uptake studies showed that TBr4 (1.88±0.11 μM) internalization is considerably higher than BBr2 (0.11±0.01 μM). Despite this difference, the cell proliferation studies showed the higher photodynamic effect of BBr2 (IC50 at 24 hours: 180 nm) in comparison to TBr4 (IC50 at 24 hours: 464 nm). The proliferation studies where the irradiation step was omitted showed that light activation is essential for cytotoxicity (IC50 at 24 hours: 7.08 μM for BBr2 and >10 μM for TBr4). Flow cytometry studies showed that both PS induced death by necrosis in the WiDr cells.

Conclusions: TBR4 and BBR2 are promising PS with good photodynamic effect in human colorectal adenocarcinoma cell lines: both localize in the mitochondria, have low IC50 when compared with reference compounds and induce cell death by necrosis in the presence of light.
Photodynamic therapy (PDT) is a modality capable of inducing cell death by oxidative stress through activation of a sensitizer by light. Photoporphins proved to be good photosensitizers, and recent works showed that addition of hydroxy groups and the presence of heavy atoms may contribute to enhance its activity. The aim of this work was to compare the photodynamic activity of 5,10,15,20-tetrakis(2-bromo-3-hydroxyphenyl)porphyrin (TBr4) and 5,15-bis(2-bromo-3-hydroxyphenyl)porphyrin (BBr2) against human colorectal adenocarcinoma.

**Material and Methods:** The molecules were synthesized as described. After proving their sensitizing action, the sensitizers have good photodynamic activity in vitro with an IC50 of 180 nM for BBr2 and 464 nm for TBr4. In vivo studies were performed. BAbc nu/nu mice were injected with 4×106 cells of human colorectal adenocarcinoma cell line WiDr in the dorsal region. When tumour volume reached 300–500 mm3, mice were administered intraperitoneally (2 mg/kg) and after 24 or 48 or 72 hours, tumours were irradiated using a laser light until a total of 180J. The control group consisted of animals irradiated with the same conditions but without administration of sensitizers. Tumour volume monitoring was carried out every 48 hours for 12 days. To analyse the prospective study Kaplan–Meier survival curves were performed that were compared using the Wilcoxon test. After 12 days tumours were excised and histopathological analysis was performed. **Results:** It was found that treatment with both sensitizers significantly reduces tumour growth. For the sensitizer BBr2 there was a decrease in tumour growth (p < 0.001) in treated mice compared to the control group. The most effective treatment is irradiation 72 hours after sensitiser administration (p = 0.006), with a significant decline in growth in just the second day of monitoring. Also at 48 hours after irradiation, there was a decrease of tumour growth (p = 0.03). For the sensitizer TBr4 was verified the existence of very significant differences between irradiated groups and control group (p < 0.009). For each irradiation time were also observed differences from the control, 24 hours: p = 0.006, 48 hours: p = 0.03 and 72 hours: p = 0.006. Although no significant differences were found between the two sensitzers, for BBr2 treatment with irradiation at 72 hours, there are five in six events on the second day of follow-up. In the analysis of tumours excised from mice were observed large areas of necrosis but also some areas of living cells. **Conclusions:** Photodynamic therapy based on BBr2 and BBr4 have the ability to induce cell death by necrosis in colorectal adenocarcinoma cells in vitro and in vivo. The results of PDT in vivo proved that both formulations are not toxic, showing no side effects and having significant photodynamic effect at relatively low doses.

**Cediranib Affects Tumor Progression and Survival of Mice Bearing Human Ovarian Carcinoma Xenografts Expressing VEGFC**

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**Background:** Vascular Endothelial Growth Factor C (VEGFC) is the main promoter of lymphatic vessel formation. It acts via the tyrosine kinase VEGF receptor-3 and -2 inducing lymphangiogenesis and tumor cell spread mainly through the lymph nodes.

**Materials and Methods:** The anti-proliferative activity of KRC-108 was measured by performing cytotoxicity assays on a panel of cancer cell lines (NCI-60). To assess in vivo anti-tumor activity, human NCI-H441 non-small-cell lung cancer and HT29 colon cancer xenograft models in nude mice were employed.

**Results:** We found that, in the neoadjuvant radio-chemotherapy group, ERCC1 8092 C/A and AA genotypes, although not associated with therapeutic response, are prognostic markers of better outcome, compared to the C/C genotype. Also, the anti-proliferative activity of KRC-108 is significantly lower in the presence of glutathione-S-transferase polymorphism.

**Conclusion:** Our study, despite its retrospective nature, strongly suggests the ERCC1 C8092A polymorphism as a possible indicator of better outcome in esophageal cancer patients who undergo neoadjuvant treatment and, once validated in further analyses, as a marker that could be taken into account for therapeutic decision making.

**KRC-108 as a Potent C-Met/ Ron/Flt3 Kinase Inhibitor with Anti-tumor Effects in Vitro and in Vivo**

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**Background:** Kinases have been studied as potential cancer targets because they play important roles in the cellular signaling of tumors. A number of small molecules targeting kinases are prescribed in clinics and many kinase inhibitors are being evaluated in the clinical phase. Previously, we discovered a series of aminopyridines substituted with benzoaxole as orally active c-Met kinase inhibitors. One of the compounds, KRC-108, has been evaluated as an antiproliferative agent in vitro and in vivo.

**Materials and Methods:** Inhibition of kinase activity against recombinant proteins were measured using homogeneous time-resolved fluorescence assays. The anti-proliferative activity was evaluated by performing cytotoxicity assays on a panel of cancer cell lines (NCI-60). To assess in vivo anti-tumor activity, human NCI-H441 non-small cell lung cancer and HT29 colon cancer xenograft models in nude mice were employed.

**Results and Discussion:** A kinase panel assay exhibited that KRC-108 is a potent inhibitor of Ron, Flt3 and c-Met as well as c-Met. Moreover, KRC-108 inhibited oncogenic c-Met M1250T and Y1230D more strongly than wild type c-Met. The anti-proliferative activity of KRC-108 was measured by performing a cytotoxicity assay on a panel of cancer cell lines. The GI50 values (i.e., 50%
inhibition of cell growth) for KRC-108 ranged from 0.01 to 4.22 (mL) for these cancer cell lines. KRC-108 was also effective for the inhibition of tumor growth in human HT29 colorectal cancer and NCI-H441 lung cancer xenograft models in athymic BALB/c nude mice.

Conclusion: This molecule should serve as a useful lead for inhibitors targeting kinases and may lead to new therapeutics for the treatment of cancer.

[942] Functional Consequences Mediated by the Inhibition of the Fanconi Anemia Pathway in Transformed Cells Treated With Anticancer Drugs of Marine Origin

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Background: Previous studies have shown that around 10% of solid and hematological tumors have a disrupted Fanconi anemia (FA) pathway either because of gene mutations or due to the silencing in any of the 15 FA genes so far discovered.

Because the FA pathway is involved in the repair of interstrand cross-links, in this study we have investigated the response of FA competent and FA-deficient cells to three anticancer DNA-binding drugs of marine origin (ADMO: trabectedin, Zalypsis and PMO11183) in different stages of clinical development.

Materials and Methods: Clonogenic assays with bone marrow samples from WT and FA-deficient mice were performed. Cell viability of human lymphoblastoid cell lines (LCLs) and of different human cancer cell line were analyzed with propidium iodide. Cell lines included the following: Acute megacarioblastic leukemia FA-CHRF28811, FA-A like acute myeloid leukemia, Kasumi1 cells transduced with anti-FANCA shRNA; FA-A and FA-C squamous cell carcinomas (SCCs), EUFA1365, EUPA1131. Mytomycin C was used as a control. Expression levels of FANCD2 were also determined by Western blot and immunohistochemistry.

Results: Hematopoietic progenitors from FANCA or BRCA2 mice were highly sensitive not only to mitomycin C (MMC) (a classical DNA cross-linker) but also to all tested ADMOs, compared to WT progenitors. A similar response was also observed in human LCLs from FA-patients when compared to samples from healthy donors analyzed by propidium iodide. Unexpectedly, the disruption of the FA pathway in different types of cancer cells (SCCs and leukemias) did not have the same impact as that observed in untransformed cells regarding their sensitivity to ADMOs. Interestingly when expression level of FANCD2, a critical protein in the FA protein, was determined in transformed and untransformed cancer cells, it was higher in MMC or to clinically-relevant concentrations of these ADMOs, a marked FANCD2 down-regulation was observed in cancer cells, but not in untransformed cells. Because the FA pathway is required for the repair of DNA cross-links, additional drug combination studies were conducted.

Conclusions: The data showed that ADMO sensitized cancer cells to MMC, strongly suggesting that the combined use of ADMOs with classical DNA cross-linking drugs, like MMC or cisplatin, would confer more potent antitumor effect in a variety of cancer cells.

[943] Study of the Combined Effect of Long-term Exposure to Pulsed Magnetic Field and Bleomycin on In Vitro Genomic DNA

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Background: Pulsed magnetic field (PMF) is a physical agent that is being studied for its harmful effects which are not fully known yet. Although many studies have observed PMF effects on living organism, the co-exposure with other genotoxic agents has not been widely investigated. Bleomycin is an antineoplastic drug that causes DNA strand breaks. The aim of this work is to investigate whether PMF exposure can induce alterations in the potency of bleomycin.

Materials and Methods: Genomic DNA: It was prepared from S. cerevisiae (WS8105-1C) cultures by phenol-chloroform extraction and ethanol precipitation. Yeast cells were grown in yeast extract-Peptone-Dextrose broth (1:2:2%), 12 μl of DNA was used in each sample. Exposure to MMS and UVC: Different concentrations of MMS (0, 0.5 and 1%), different UVC (253.7 nm) doses (0, 25 and 50 J/m²) and different time of exposure to MMS were assayed. Three experimental groups were studied: (1) samples exposed 60 minutes to MMS and then UVC exposures; (2) Samples exposed 24 hours to MMS and then UVC exposures; and (3) 48 hours of exposure to MMS and then UVC treatments. Equal environmental conditions were maintained for all samples.

Aposear gel electrophoresis: After treatments, DNA samples were electrophoresed in a 1% agarose gel (in 0.5 X Tris-Borate EDTA) at 80 V, 90 min. The gel was stained with ethidium bromide 10 mg/ml. After electrophoresis, photography was taken from each gel and the images were analyzed.

Results: The first treatment group (60 minutes of exposure to MMS), did not show DNA degradation after UVC and/or MMS exposures. However the groups exposed 24 and 48 hours to MMS showed an increased DNA degradation, dose and time dependent, that was very intense at 48 hours of exposure to MMS. Moreover, the combined exposure to MMS and UVC showed and additional increase in DNA degradation in the groups exposed to MMS (48 hours; 0.5 and 1%) and then exposed to 24 J/m² of UVC.

Conclusion: The data obtained in this investigation show that UVC radiation (24 J/m²) in combination with MMS (48 hours; 0.5 and 1%) have the ability to augment the potency of MMS in the induction of DNA degradation.

[944] Ultraviolet C Radiation Increases the DNA Degradation Induced by Methyl Methane Sulphonate

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Background: It is well known that pre-exposure to a mild stressor can confer protection against a subsequent lethal agent (preconditioning effect). The primary damage from exposure to ultraviolet C (UVC) is the damage induced to the DNA molecule due to its maximum light absorption (240–290 nm). In addition, UVC exposures can lead to the formation of reactive oxygen species, which are responsible for causing secondary damage. Methyl methane sulphonate (MMS) is a genotoxic agent that causes DNA damage by the alkylation of nucleotidic bases. So far, there have been very few studies looking for chemosensitizing agents. The aim of this work was to investigate whether UVC radiation produce alterations in the DNA degradation induced by MMS.

Materials and Methods: Genomic DNA: It was prepared from yeast (S. cerevisiae haploid strain WS8105-1C) cultures by phenol-chloroform extraction and ethanol precipitation. Yeast cells were grown in yeast extract-Peptone-Dextrose broth (1:2:2%, 12 μl of DNA was used in each sample. Exposure to MMS and UVC: Different concentrations of MMS (0, 0.5 and 1%), different UVC (253.7 nm) doses (0, 25 and 50 J/m²) and different time of exposure to MMS were assayed. Three experimental groups were studied: (1) samples exposed 60 minutes to MMS and then UVC exposures; (2) Samples exposed 24 hours to MMS and then UVC exposures; and (3) 48 hours of exposure to MMS and then UVC treatments. Equal environmental conditions were maintained for all samples.

Aposear gel electrophoresis: After treatments, DNA samples were electrophoresed in a 1% agarose gel (in 0.5 X Tris-Borate EDTA) at 80 V, 90 min. The gel was stained with ethidium bromide 10 mg/ml. After electrophoresis, photography was taken from each gel and the images were analyzed.

Results: The first treatment group (60 minutes of exposure to MMS), did not show DNA degradation after UVC and/or MMS exposures. However the groups exposed 24 and 48 hours to MMS showed an increased DNA degradation, dose and time dependent, that was very intense at 48 hours of exposure to MMS. Moreover, the combined exposure to MMS and UVC showed and additional increase in DNA degradation in the groups exposed to MMS (48 hours; 0.5 and 1%) and then exposed to 24 J/m² of UVC.

Conclusion: The data obtained in this investigation show that UVC radiation (24 J/m²) in combination with MMS (48 hours; 0.5 and 1%) have the ability to augment the potency of MMS in the induction of DNA degradation.
Molecular Profiling of Residual Tumor Cells After Various Chemotherapeutic Treatments Shows Distinct Gene Expression Patterns in Patient-derived Breast Cancer Xenografts


Introduction:
The characterization of tumor cells responsible for recurrences after various chemotherapeutic treatments using patient-derived TNBC xenografts (HBCx-10 and HBCx-33).

Material and Methods:
Two xenograft models showing pathological tumor remission after chemotherapy followed by local relapse were treated with AC (adriamycin + cyclophosphamide) (HBCx-10) or with cisplatin or capetacitabine (HBCx-33). RNA was collected from pre-chemotherapy tumors (untreated group), from residual cancer cells after chemotherapy (remission group) and from relapsing tumors (recurrence group). Gene expression profiles were obtained using Affymetrix U133 Plus 2.0 Arrays and RT-PCR approaches. In the tumor remission group, samples having at least 30% of human residual cancer cells (RCC) were analyzed. Genes having one or more probesets cross-reaction between human and mouse were removed from the analysis.

Results:
Exploratory analyses of expression datasets revealed the existence of two distinct groups: one consisting of untreated and relapsed tumors and the other of RCC. The differential analysis showed no difference in gene expression between untreated and relapsed tumors in the 2 models analyzed, whatever the cytotoxic agent administered. In contrast, when gene expression was analyzed in RCC compared to untreated or relapsed tumors, we found 594 genes differentially expressed in the AC-treated HBCx-10 xenograft (fold change greater than 3 or lower than −3), and 1011 and 241 genes in the HBCx-33 model treated with capetacitabine and cisplatin, respectively. Among these 3 lists, we found 73 common differentially expressed genes. Gene Ontology analysis of these genes revealed enrichment in several biological processes, including cell migration, immune response, response to wounding, inflammatory response and cell adhesion. An RT-PCR analysis confirmed the expression changes of a set of genes with human breast tumor origin and highlighted a strong up-regulation of several interferon-responsive genes in RCC.

Conclusions:
This study supports the existence of a distinct molecular profile of residual tumor cells after chemotherapy, with a subset of deregulated human genes common to the different tumors analyzed and the different treatments administered. Further elucidation of the signaling pathways associated with these deregulated genes may provide key insights into the mechanisms driving breast cancer recurrence and potential therapeutic targets.

Transcriptome Analysis and Theranostic Profiling of Ewing Sarcoma (ES) and Desmoplastic Small Round Cell Tumours (DSRCT)

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Background:
Ewing Sarcoma (ES) and Desmoplastic Small Round Cell Tumor (DSRCT) are sarcomas with distinct chromosomal translocations involving the EWS gene (predominately EWS-FLI1 and EWS-WT1; respectively). Their diagnosis and treatment have been difficult due to the rarity, diverse clinical presentation, overlapping histologic features and genetic complexity. Identification of therapeutically targetable genes or pathways in distinct tumor group and individual patient might provide more effective therapeutics strategies.

Materials and Methods:
Twenty cases (13 ES and 10 DSRCT) were analyzed using a molecular profiling service (CarisTargetNow™, Caris Life Sciences, Phoenix, AZ). The whole genome transcriptome analysis (29285 transcripts) was performed using the CarisTargetNow™ platform. Translational Research, Paris, France, 3Institut Curie, Bioinformatics, Paris, France, 4Institut Curie, Oncogenetics, St-Cloud, France

Conclusion:
The potency of MMS (0.8%) on DNA degradation is enhanced by long-term co-exposure with PMF (1.5 mT, 25 Hz, 48 hours). PMF could be a potential chemosensitizing agent.

Molecular Profiling of Residual Tumor Cells After Various Chemotherapeutic Treatments Shows Distinct Gene Expression Patterns in Patient-derived Breast Cancer Xenografts

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Background:
Triple negative breast cancer (TNBC) is an aggressive disease associated with a high risk of distant recurrence, rapid progression to death and poor overall survival. Systemic treatments of TNBC patients are limited to cytotoxic chemotherapy given due to the lack of identified molecular targets. Therefore, the characterization of tumor cells responsible for recurrences after such treatments is of high therapeutic relevance. We therefore examined gene expression of residual cancer cells (RCC) before and during relapse after various chemotherapeutic treatments using patient-derived TNBC xenografts (HBCx-10 and HBCx-33).

Material and Methods:
Two xenograft models showing pathological tumor remission after chemotherapy followed by local relapse were treated with AC (adriamycin + cyclophosphamide) (HBCx-10) or with cisplatin or capetacitabine (HBCx-33). RNA was collected from pre-chemotherapy tumors (untreated group), from residual cancer cells after chemotherapy (remission group) and from relapsing tumors (recurrence group). Gene expression profiles were obtained using Affymetrix U133 Plus 2.0 Arrays and RT-PCR approaches. In the tumor remission group, samples having at least 30% of human residual cancer cells (RCC) were analyzed. Genes having one or more probesets cross-reaction between human and mouse were removed from the analysis.

Results:
Exploratory analyses of expression datasets revealed the existence of two distinct groups: one consisting of untreated and relapsed tumors and the other of RCC. The differential analysis showed no difference in gene expression between untreated and relapsed tumors in the 2 models analyzed, whatever the cytotoxic agent administered. In contrast, when gene expression was analyzed in RCC compared to untreated or relapsed tumors, we found 594 genes differentially expressed in the AC-treated HBCx-10 xenograft (fold change greater than 3 or lower than −3), and 1011 and 241 genes in the HBCx-33 model treated with capetacitabine and cisplatin, respectively. Among these 3 lists, we found 73 common differentially expressed genes. Gene Ontology analysis of these genes revealed enrichment in several biological processes, including cell migration, immune response, response to wounding, inflammatory response and cell adhesion. An RT-PCR analysis confirmed the expression changes of a set of genes with human breast tumor origin and highlighted a strong up-regulation of several interferon-responsive genes in RCC.

Conclusions:
This study supports the existence of a distinct molecular profile of residual tumor cells after chemotherapy, with a subset of deregulated human genes common to the different tumors analyzed and the different treatments administered. Further elucidation of the signaling pathways associated with these deregulated genes may provide key insights into the mechanisms driving breast cancer recurrence and potential therapeutic targets.
Poster Sessions

**Molecular Studies of Mechanisms of Drug Resistance in Malignant Cells With Focus on Lymphomas**

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**Background:** Multidrug resistance to anticancer drugs is a major cause of chemotherapy failure in cancer patients. In efforts to find novel approaches to inhibit proliferation and induce apoptosis in lymphoma cells, we examined in both Hodgkin and non-Hodgkin lymphoma cell lines, the action of naturally occurring compound curcumin which is nontoxic and has a variety of therapeutic properties including anti-oxidant, analgesic, anti-inflammatory and antiangiogenic activity.

**Methods:** Both Hodgkin and non-Hodgkin cells were pre-treated with curcumin followed by exposure to doxorubicin or vincristine and the effect on cell growth was determined. Cytotoxic effects and determination of apoptotic attributes upon curcumin treatment were analyzed using flow cytometry assays.

**Results:** The current study demonstrates that curcumin has the ability to decrease cell viability and it is due to its capacity to decrease cell proliferation by causing cell cycle arrest in G2/M phase, and by inducing apoptotic cell death. The pre-treatment followed by exposure to doxorubicin or vincristine increased apoptosis as indicated by annexin V staining. It is shown that curcumin is much more effective on lymphoma cell lines in compare to doxorubicin or vincristine.

**Conclusion:** We have demonstrated that curcumin is an efficient inducer of apoptosis in lymphoma cell lines, meriting its further evaluation in vivo. The observed effects combined with the well established pharmacological safety of curcumin, provides rationale for the potential use of curcumin as a new therapeutic agent for patients with Hodgkin and non-Hodgkin lymphomas.

**Uptake and Immunomodulatory Effect of Pegylated Liposomal Doxorubicin Nanoparticles on Human Macrophages**


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**Introduction:** The pegylated liposomal doxorubicin (PLD) has been widely accepted in treatment of various cancers. However, the composition of two currently marketed PLD nanoparticles differs in structure and composition of lipids, and their differential effects remain unknown. Macrophages of the mononuclear phagocyte system are pivotal in determination of PLD clearance in vivo. The aim of this study was to compare the effect of these two PLDs on drug uptake, cell viability, morphology and immune function of human macrophages.

**Material and Method:** Two PLD nanoparticles were used in this study. The major difference between Lipo/dox (PLD-D) and Caelyx (PLD-H) is that their phospholipid bilayers are composed of distearoyl phosphatidylcholine (DSPC) and hydrogenated soybean phosphatidylcholine (HSPC), respectively. Human CD14+ monocytes were isolated from peripheral blood to prepare macrophages for this study. Comparative assays included: flow cytometry for detection of doxorubicin penetration into cells, MTT for cell viability, Trypan blue exclusion for cell membrane integrity, Liu’s stain for morphologic evaluation, inactivated yeast co-culture for phagocytosis.

**Results and Discussion:** The uptake of PLD-D was rapidly detected at 10 min and kept increasing to 4 h followed by a decline thereafter, whereas that of PLD-D had similar profile with much less doxorubicin fluorescence detected, indicating a greater amount of doxorubicin retention of PLD-D. PLD-H, at higher concentration, decreased the viability and impaired cell membrane integrity of macrophages with an extent greater than PLD-D. The morphological observation showed a more extensive necrosis in PLD-H treated macrophages. The phagocytosis function of macrophage was inhibited with a greater effect in PLD-H-treated macrophages.

**Conclusion:** To human macrophages, PLD containing HSPC may cause greater amount and longer retention of doxorubicin in cells, greater toxicity and more profound dysfunction than that containing DSPC. Whether this differential effect correlates to the clinical outcome needs to be extensively investigated by performing in vivo experiments or clinical trials.

**Discovery of a New Inhibitor of P53/MDM2 Interaction Using a Yeast Target-based Screening Strategy**

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**Introduction:** Ewing Sarcoma (ES) is a malignant tumor affecting mainly children and young adults, especially in bone and soft tissue and characterized by the presence of a chromosomal translocation responsible for the transcriptional deregulation of target genes such as the membrane receptor CD99. The origin of ES has long been the focus of intensive research, however, recently a Mesenchymal Stem Cell (hMSC) origin has been assumed as the most probable. Despite prior evidence suggesting a hMSC origin of ES, hMSC from ES patients have not been sufficiently investigated. Herein, we compared a large
set oh hMSC and ES cell lines. In addition, the expression of CD99, together with the cytotoxic effects derived from its engagement were specifically evaluated in hMSC derived from a plurality of tissues and different benign bone lesions.

**Material and Methods:** We studied several hMSC (n = 44) derived from the Bone Marrow (BM) of Healthy Donors (HD), ES Patients (ES-P) and patients with other biding lesions. We also studied hMSC derived from tissues other than BM, such as Adipose Tissue, Placenta, Dental Pulp, Amniotic and Chorion Membrane. We also studied ES cell lines (n = 9). Samples were studied using iFISH analysis and Multiparameter Flow Cytometry assays.

**Results:** The primary aim of our study was to determine whether BM-hMSC derived from ES patients shared molecular elements in common with ES cells. Initially, we observed that hMSC-ES-P lack the presence of the EWS-FLI1 gene fusion, in contrast to ES cells. In light of this, we analyzed the presence of other ES markers. Our results suggest that there is no evident differential expression patterns between hMSC-ES-P and hMSC-HD, regarding the ES surface markers CD271, CD54 and CD117. Conversely, ES presented some isolated hMSC features.

Moreover, we conducted a detailed study of CD99 expression in hMSC from different bone lesions and normal tissues, given that this particular antigen has been suggested to be determinant for ES malignancy and potential therapeutic target for ES cells. Our findings demonstrated that hMSC from different patients (ES-P or patients with unrelated benign bone diseases) and HD show lower expression of CD99 in comparison to ES cells.

Finally, we found that hMSC displayed an expression level of CD99 similar to that of ES cells were unable to undergo apoptosis when exposed to the anti-CD99 murine antibody 0862.

**Conclusions:** In conclusion, (i) hMSC derived from ES Patients behave similarly to hMSC from HD and fail to present differential expression of ES markers; (ii) EWS cells present some similarities to MSC although its global phenotype is distinct from MSC; (iii) hMSC derived from other bone lesions and normal tissues present variable levels of CD99 and (iv) 0862 is able to induce massive cell death in ES cells while maintaining MSC viability and integrity. CD99 targeting may be considered as a highly promising therapeutic approach in ES, presenting high cytotoxic activity towards ES cells and a high safety profile towards hMSC.

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**R55 Antitumor Effects of Retinoic Acid Combined With Antiestrogens on Melanoma Cells**

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**Introduction:** Malignant melanoma remains among the most notoriously aggressive and treatment-resistant of human cancers. Emerging evidence points to the involvement of multiple signaling pathways, rather than the existence of a crucial individual alteration. Therefore, we need to develop combinations of drugs with specific and complementary mechanisms of action in order to increase treatment efficacy, with less toxic effects. Retinoid acid (RA), the most abundant natural retinoid, has been shown to inhibit the growth and survival of melanoma cells. However, the clinical utilization of retinoids has been limited by their adverse effects.

Recent work has pointed to the interplay between retinoid and estrogen signaling in breast cancer and the combination of retinoids with antieostrogens has revealed a synergistic action in breast cancer cells.

Tamofoxifen (Tam) is a pro-drug that is activated to 4-hydroxytamoxifen (OHTam) and endoxifen (EDX), by cytochrome P450 (CYP) enzymes, which present therapeutic advantages comparing with the use of Tam, since their efficacy is not limited by variable CYP enzymes activity. Tam has been used routinely in the treatment of breast carcinoma due to its action on estrogen receptor (ER), but it also suppresses tumor cell growth, independent of the expression of ERs, through induction of apoptosis and angiogenesis inhibition. Moreover, it has been reported that Tam is an effective inhibitor of the growth and invasion of melanoma cells. Therefore, we aimed to evaluate the effects of a combined therapy of RA with TAM and its active metabolites, OHTAM and EDX, on a melanoma cell line.

**Materials and Methods:** Cell proliferation was measured by sulforhodamine B (SRB) and bromodeoxyuridine (BrDU) incorporation assays, cell death by the lactate dehydrogenase (LDH) assay and by cell count with trypan blue staining.

**Results and Discussion:** The combinations of low concentrations of RA with antieostrogens exhibited additive antiproliferative effects on melanoma cells, as shown by SRB assays. The effect on tumor cell proliferation is not due to increased cell death, since the combinations did not enhance LDH release neither trypan blue positive cells. Measurements with incorporation of BrDU suggest that RA and antieostrogens additively decrease cell division.

**Conclusion:** Altogether, these findings support a combined therapy of RA and antieostrogens as a promising strategy for melanoma treatment.

This study was supported by a PhD grant (SFRH/BD/65130/2009) attributed to M.P.C. Ribeiro by FCT.
Conclusions: Spectrum of KRAS mutations in CRC from Russian patients is specific. KRAS mutation analysis may help to identify CRC patients who are sensitive to the antitumor antibodies, which makes it possible to personalize the molecular-directed therapy of patients with metastatic colorectal cancer.

**[95]** The MEK Inhibitor BAY 86-9766 Prolongs Survival in Orthotopic, Syngeneic Animal Models of Hepatocellular Carcinoma and Pancreatic Cancer

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Introduction: BAY 86-9766 (RDEA119) is an orally available, allosteric MEK1/2 inhibitor which is currently in clinical phase I/II studies in hepatocellular carcinoma (HCC) and pancreatic cancer (PaCa) indications. BAY 86-9766 exhibited potent anti-proliferative activity across multiple cancer cell lines in vivo and in vitro. BAY 86-9766 was studied predilection in two orthotopic, syngeneic HCC and PaCa models and five subcutaneous PaCa xenograft models as a single agent and in combination therapy. In contrast to subcutaneous xenograft models orthotopic, syngeneic models enable organ specific interaction with tumor cells. Additionally, they show metabolic spread and provide important information on the immune response and survival.

Results: BAY 86-9766 was first evaluated in vitro in the rat HCC cell line MH929/4a and exhibited anti-proliferative effects with an IC50 value of 238 nM. In combination with Sorafenib strong synergistic effects were detected. This synergistic activity, as well as anti-proliferative effects were confirmed in the corresponding orthotopic, syngeneic HCC rat model. Primary tumor growth was significantly reduced. These effects were accompanied by a reduction in pERK levels, proliferation index and microvessel density. Finally, the median overall survival was doubled in animals treated with the combination of sorafenib and BAY 86-9766 compared to vehicle.

Conclusion: Combination of BAY 86-9766 in an orthotopic syngeneic PaCa model, the Panc02 mouse allograft model was established. BAY 86-9766 was tested in mono- and combination therapy with Gemcitabine. Combination treatment led to a significantly lower tumor weight, reduction of metastatic spread and an increase in median survival of 135%.

In five patient-derived PaCa models tumor samples were subcutaneously implanted in mice and subsequently treated with BAY 86-9766. Significant tumor growth inhibition in these xenograft models was observed.

Conclusion: BAY 86-9766 is efficacious in orthotopic, syngeneic animal models of HCC and pancreatic cancer. In combination with standard of care drugs both additive and synergistic effects on tumor growth were seen. These results support the further development of BAY 86-9766 in the indications HCC and PaCa.

**[96]** Combining Vascular Disruption Activity of TRAIL-expressing CD34+ Cells With Antiangiogenic Activity of Sorafenib Improves Regression of Tumor Growth

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Introduction: We have demonstrated that adenosine-transduced CD34+ cells expressing membrane-bound TRAIL (CD34-TRAIL+ cells) act in vivo as a vascular disrupting agent (VDA). Intravenous injection of CD34-TRAIL+ cells induces apoptosis of tumor endothelial cells causing vascular disruption, hemorrhagic necrosis and tumor destruction. Combining VDAs with antiangiogenic agents might result in greater antitumor efficacy than is achievable with either treatment alone. We therefore investigated the antitumor efficacy of CD34-TRAIL+ cells in combination with the antiangiogenic agent sorafenib.

Material and Methods: Human CD34+ cells were transduced with an AdSF35 adenosine encoding the human TRAIL gene. In vivo experiments were performed in NOD/SCID mice bearing subcutaneous human multiple myeloma (KMS-11 cell line). In vivo biodistribution of endothelial cells with sialo-NHS-LC-biotin allowed to visualize functional vessel network and to perform 3D reconstruction of tumor vasculature. TUNEL staining was performed to detect tumor necrosis. ImageJ software was used to quantify vessel density and tumor necrosis within endothelial sections.

Results and Discussion: CD34-TRAIL+ cells and sorafenib alone reduced KMS-11 tumor growth by 38% (p < 0.001). Combining CD34-TRAIL+ cells with sorafenib reduced tumor volumes by 61% (p < 0.001) compared with controls and antiangiogenic treatment alone. After single-tranlated tumor nodules revealed a 35% (p < 0.001) and 42% (p < 0.001) reduction of endothelial overall survival following treatment with CD34-TRAIL+ cells and sorafenib, respectively. The cotreatment further reduced tumor endothelial area by 63% (p < 0.001) over control, by 43% (p < 0.001) over CD34-TRAIL+ cells and 37% (p < 0.001) over sorafenib. 3D reconstruction of KMS-11 vessel network showed a more damage in tumor vasculature lacking branches and sprouts following treatment with CD34-TRAIL+ cells plus sorafenib compared with single agents. Reduction of tumor vascularization significantly amplified tumor necrosis. Compared with controls, the combined treatment increased tumor necrosis by 16-fold (p < 0.001), whereas treatment with CD34-TRAIL+ cells and sorafenib alone increased tumor necrosis by 6- (p < 0.01) and 4-fold (p < 0.05), respectively.

Conclusions: Our results demonstrate that the antitumor efficacy of CD34-TRAIL+ cells can be widely improved combining their vascular disruption activity with the antiangiogenic activity of the multikinase inhibitor sorafenib.

**[97]** Optimization of Molecular Diagnostic Genotyping Strategies

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Background: More and more targeted drugs become available increasing the need to stratify patients based on the genetic profile of the tumor cells. Challenges in molecular diagnostics are: availability of results within days, accuracy, clinically relevant sensitivity, and robust performance using small FFPE biopsies and cytological material. To overcome the limitations of Sanger sequencing approaches we tested two novel genotyping strategies: TaqMan® Mutation Detection Assays based on Competitive Allele-Specific PCR technology (cASPCR, LifeTechnologies) and an amplicon-based next generation sequencing (NGS) technology using the Ion PGM™ system.

Material and Methods: Formalin-fixed paraffin-embedded (FFPE) patient material was obtained from the archives of the departments of pathology at the Radboud University Medical Center Nijmegen. Extracted and purified DNA was used for KRAS TaqMan® Mutation Detection Assays analysis and amplicon-based NGS (Ion Torrent), according to manufacturer's instructions. Results were compared with Sanger sequence analysis applying GOLD-PCR in cases of low tumor cell percentages.

Results: The TaqMan® Mutation Detection Assays allows fast, accurate and sensitive identification of KRAS mutations in colon and lung FFPE tissue. After DNA purification experimental setup, PCR and data analysis take less than 3 hours. All KRAS point mutations present in the tested tissues were readily identified with a sensitivity of 5% tumor cells. Rare KRAS double mutations are missed, however these 'errors' remain currently without clinical consequences. Since all mutations are assessed in individual PCR reactions, the limiting factor is the amount of DNA required, especially when little tissue is available for analysis. Preliminary NGS data show that this approach allows for sensitive analysis of mutant genes using little tissue within 2 days. The possibility to correlate the percentage of mutated alleles with the tumor cell percentage makes it possible to assess the heterogeneity of the tumor and will eventually improve treatment choices.

Conclusions: Depending on the diagnostic setting and clinical questions the TaqMan® Mutation Detection Assays offer fast and reliable data for personalized treatment decisions, however with the increasing need for the simultaneous analysis of multiple genes, NGS will probably become the method of choice in the near future.

**[98]** Effects of Different Types of Hsp90 Inhibitors in Pancreatic and Colorectal Cancer Cell Lines

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Background: The use of HSP90 inhibitors has emerged as an attractive antineoplastic therapy in the last two decades because the chaperone heat shock protein 90 (HSP90) participates in the folding, maturation and activation of numerous oncoproteins. We wanted to determine the effects of these groups of HSP90 inhibitors: the ansamycin class by using geldanamycin (GA) and its derivate 17-allylamino-17-demethoxygeldanamycin (17-AAG), and the resorcinol class by using radicicol and the potent and novel synthetic compound NVP-AUY922. Both types of drugs bind to HSP90-terminal adenosine triphosphate (ATP)-binding pocket of HSP90 blocking its activity and they have been proposed as a possible chemotherapeutic alternative for those tumors that show resistance to current treatments.

Materials and Methods: We have investigated the effect of these HSP90 inhibitors in cell proliferation assays, cell cycle distribution of DNA content measured by flow cytometry, Western blot analyses, and enzymic activity assays in a panel of five human colorectal and pancreatic cancer cell lines.

Results: We show that cell lines are growth inhibited in vitro when treated with 0.1 μM NVP-AUY922 or 0.5 μM GA for 6 days. The pancreatic cell lines CFPAC-1, PAN-1 and IMIM-PC-2 and the colorectal cell line Caco-2 are
resistant to 0.5 μM 17-AAG in cell proliferation assays, whereas IMIM-PC-2 is the only cell line resistant to 0.5 μM radicicol. In general, these inhibitors induce a G2/M cell arrest, except in IMIM-PC-2 cells, which are arrested in the G2 phase of the cell cycle. Especially, HT-29, SW-620 and IMIM-PC-1 cells accumulate in G2/M, followed by an increase in subG1. Additionally, we have studied the relationship between the presence of the enzyme quinone oxireductase 1 (NQO1) and 17-AAG activity, because previous studies have shown that this enzyme metabolizes the quinone 17-AAG to hydroquinone (17-AAGH2), which has more affinity for HSPP90. However, the level of NQO1 in the sensitive cell lines is controversial, the enzyme is present in IMIM-PC-2 cells that are resistant to 17-AAG vs. the other sensitive cell lines.

Conclusions: There are three cell lines especially sensitive to HSPP90 inhibitors: HT-29, SW-620 and IMIM-1. Furthermore, our study shows that NVP-AUY922 treatment is the most effective HSPP90 inhibitor in pancreatic and colorectal carcinoma cell lines. Therefore, NVP-AUY922 is the best candidate to be used in a clinical setting.

**555 Effects of Chemotherapeutic Drugs in Colorectal Cancer Cells**

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**Background:** Colorectal carcinoma is a very aggressive and common type of tumor. 5-Fluorouracil (5-FU), capecitabine, prodrug of 5-FU, irinotecan and oxaliplatin are the first line of chemotherapy drugs used in colorectal cancer. We have used a panel of colorectal cancer cells to determine the effects of these drugs in cell proliferation, cell cycle and induction of DNA damage. We also studied the response to these treatments using primary cell cultures obtained from tumors excised from patients.

**Materials and Methods:** In order to determine the degree of sensitivity/resistance of carcinoma colon line cells and primary cell cultures to chemotherapeutic drugs, we used cell proliferation assays. We utilized flow cytometry to determine cell cycle distribution of DNA content and to assess DNA damage by accumulation of reactive oxygen species (ROS) and increase in phosphorylation levels of (S139) histone h3TAX.

**Results and Discussion:** We have observed that the colorectal cancer lines H181-1 and Caco-2 were resistant to 10 μM 5-FU, whereas HCT-15 and DLD-1 cell lines were resistant to 10 μM oxaliplatin. However, all of them were sensitive to 5 μM irinotecan. We also tested these drugs in primary cell cultures from tumors. The sensitive cell lines accumulated in the S phase (or G2/M) of cell cycle, followed, in some cases, by an increase in SubG1, a hallmark of apoptosis. Moreover, chemotherapeutic drugs were able to induce DNA damage in the sensitive cell lines, as demonstrated by an increase in ROS and in phosphorylation of histone h3TAX. Resistant or non-responding cell lines did not accumulate in SubG1 and do not show DNA damage.

**Conclusion:** We have ranked the cell lines according to the sensitivity/resistance to different drugs used in the clinic and found differences among them. H181-1 and Caco-2 cells are resistant to 5-FU and HCT-15 and DLD-1 cells are resistant to oxaliplatin. All cell lines studied are sensitive to irinotecan. Experiments are underway to determine mechanisms of resistance to 5-FU and oxaliplatin. We hope to find cellular determinants of sensitivity/resistance to chemotherapeutic drugs that could be used to predict the response of patients with colorectal cancer to such drugs.

**556 Modulation of Autophagic Flux by CPTH6, a Gcn5pCAF Histone Acetyltransferase Inhibitor With Antitumoral Activity**

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**Background:** The thiazole-derivative 3-methylcyclopentilidene-[4-(4′-chlorophenyl)-thiazol-2-yl]hydrazide (CPTH6) is a Gcn5 and pCAF histone acetyltransferase inhibitor that induces apoptosis and cell cycle arrest in human acute myeloid leukemia (AML) cells. The aim of this study was to evaluate whether CPTH6-induced apoptosis is associated with other cell death mechanisms, such as autophagy.

**Materials and Methods:** Human AML and a panel of solid tumor cell lines were used to evaluate the effect of CPTH6 on autophagy, as well as effects on transduction signaling pathways. Autophagy was evaluated in cells in terms of microtubule-associated protein 1 light chain 3-II (LC3-II) protein expression and localization, protein expression of autophagy promoting proteins accumulation of the autophagy substrate p62/SQSTM1. Bafilomycin A1, a proton ATPase inhibitor was used for monitoring autophagic flux in AML cells that were applied to examine toxicity, pharmacokinetic parameters and antitumor activity of CPTH6.

**Results:** Herein, we show that CPTH6 interferes with autophagic flux in several human tumor cell lines of different origin. CPTH6 treatment increases LC3-II levels and induces the appearance of typical LC3-II-associated autophagosomal puncta in a time- and dose-dependent manner. Strikingly, combined treatment of CPTH6 with Bafilomycin A1 indicates that CPTH6 reduces autophagosomes turnover, through an impairment of their degradation pathway, rather than enhancing autophagosomes formation. According to these results, CPTH6 treatment enhances p62/SQSTM1 protein levels, suggesting a block of autophagic degradation. CPTH6 also reduces the phosphorylation of several components of transduction signalling pathways, such as Akt, 4E-BP1 and eIF4E, ERK1/2, GSK-3β and it activates p38 MAP kinase. In vivo, CPTH6 exposure does not produce any adverse effects on health as mice were monitored by diet consumption, body-weight loss, postural and behavioral changes. Most importantly, CPTH6 significantly delayed tumor growth in a dose-dependent manner of human leukemia xenografts.

**Conclusions:** These findings demonstrate that CPTH6 induces apoptosis and interferes with autophagic flux in human cancer cells, supporting further exploration of CPTH6 and its derivatives as potential anticancer agents.

**561 Rapid Disturbance of the Intracellular Redox Balance Predicts Sensitivity to the Preclinical Anticancer Ruthenium Compound KP1339**

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**Introduction:** KP1339 trans-[(tetrachlorobis(1H-indazole)ruthenate(III)) is a ruthenium-based compound with promising anti-cancer activity in early clinical trials. Investigating an in vitro screening of several cancer cell lines with pronounced and drug-specific hyper-sensitivity to KP1339 were discovered. The aim of this study was to comparatively analyze gene expression responses to short-term KP1339 exposure in dependence on drug sensitivity to gain deeper insights into the mechanisms determining KP1339 response.

**Materials and Methods:** To this end, total mRNA was isolated from two hyper-sensitive and comparably resistant tumor cell lines of different cancer origin after KP1339 treatment (3 and 6 h). Gene expression arrays were performed using 4x44K whole genome oligonucleotide-based gene expression microarrays (Agilent). ROS generation was analyzed by DCF-DA staining. Changes in protein expression were investigated by Western blotting.

**Results and Discussion:** Whole genome gene expression analysis and data mining for differentially expressed genes revealed that KP1339 treatment led to the induction of genes involved in cellular redox balance predominantly in the hyper-responsive cell models. Notably, several genes such as thioredoxin 2 (TXN2) and glutathione peroxidase (GPX1) were induced upon KP1339 treatment only in sensitive cell lines. In addition, mRNAs which encode for the ROS detoxifying enzymes thioredoxin reductase 1 (TXNRD1) and xanthine dehydrogenase (XDH) were down-regulated in hyper-sensitive and up-regulated in comparably resistant cell lines. In accordance to this data, DCF-DA staining revealed stronger ROS generation in sensitive cancer cells in response to the ruthenium drug. In addition, KP1339 treatment induced a pronounced activation of SAP/JNK pathway in sensitive cell lines which was prevented by the known radical scavengers ascorbic acid and N-Acetyl cysteine.

**Conclusion:** Taken together our data reveal that KP1339 hyper-sensitivity might be associated with enhanced vulnerability to cellular redox disturbance, a characteristic of multiple cancer types.

**567 Implication of the Reactive Oxygen Species Species in the Signaling Pathway of Angiogenesis and Their Role in the Monitoring of the Antiangiogenic Treatment Efficiency**

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**Background:** Oxidative stress can activate numerous intracellular signaling pathways via ROS-mediated modulation of various enzymes and critical transcription factors. The targeted antiangiogenic treatment use monoclonal antibodies that can destroy some types of cancer cells by block the VEGF receptors, which may stop the cells from growing, or prevent the cancer cells from developing a new blood vessel. The aim of our experimental in vivo study is to establish the mechanisms when the tumor cells switches to
an angiogenic phenotype by changing the local equilbrium between positive and negative regulators of angiogenesis, starts to grow rapidly and becomes clinically detectable, also to identify if the free radicals of oxygen plays an important role in the signalling pathway for the angiogenic transformation.

Materials and Methods: We use RS1 experimental hepatoma bearing rats treated with Bevacizumab 5mg/body weight, weekly for 3 months and in this dynamics we determine the cell apoptosis by flow cytometry measurement and also the free radical of oxygen production by biochemical assays (lipid peroxidation, total thiol groups and the ability of plasma to reduce the iron).

Results: Our results show a decrease of free radical production with 15%, 23% and 29% during the treatment suggesting the inhibition of the new blood vessel formation and the oxygen apport comparitive to the untreated group. The histograms type graphics representing the DNA quantity distribution and the total cell number obtained by apoptosis measurement indicate an increase of apoptotic status with 9%, 12%, 20%, 14% and 31, 15% during the three month treatment.

Conclusions: The anti-angiogenic treatment induces apoptosis – possible-mediated by free radicals of oxygen in a concentration dependent manner. When the reactive oxygen species are in a small concentration they can initiate the cell phenotype transformation to an angiogenic form, and when there is an increase of oxygen reactive species it is optimal to administer a targeted anti-angiogenic treatment to induce the tumoral cells apoptosis.

959 Pharmacogenetic Predictors for Treatment Outcome of Sunitinib in Metastatic Renal Cell Carcinoma

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Background: The aim of this study was to investigate the pharmacogenetic determinants of sunitinib-related treatment outcome in metastatic renal cell carcinoma (mRCC) among Korean patients.

Methods and Results: A pharmacogenetic study was performed in 65 patients with mRCC treated with sunitinib (50 mg orally once daily for 4 weeks-on,2 weeks-off). Detailed data regarding the treatment outcome of sunitinib, including overall survival (OS) and progression free survival (PFS) were retrospectively collected in a Korean setting in patients with mRCC treated with sunitinib (n=28) or placebo (n=38) or general oncology practice (n=27) using sunitinib. A total of 12 genetic polymorphisms in 8 candidate genes (CYP1A1, CYP3A5, ABCG2, ABCB1, VEGFR2, RET, and FLT3) were analyzed for an association with treatment outcome.

Results: All 65 patients (100%) received at least 1 dose of sunitinib, and 62 (95.3%) patients received more than 1 cycle of sunitinib. With the median 23.8 (range 1.1–57.9) months of follow up, the median number of treatment cycle was 7 (range 1–38). Main reasons for drug discontinuation were disease progression (n=48, 73.8%) and treatment-related toxicity (n=3, 4.7%). 2 patients with both grade 3–4 hematological and non-hematological toxicities, 1 with CNS, and 1 with drug-related toxicities (dyspepsia) were enrolled in a long-term study (median follow up: 36 months). Response evaluation was performed using soft agar colony assay. SA-beta-Galactosidase assay. The effect of salinomycin on colony growth was measured using soft agar colony assay.

Conclusions: Among 12 genetic polymorphisms, c.2071 G>A in the RET gene and c.1432 T>C in the PDGFRA gene may be associated with the treatment outcome of sunitinib in mRCC patients. A better understanding of the genetic determinants of sunitinib response would help to optimize personalized drug treatment.

960 Antitumor Activity of NPS-1034, a Synthetic MET and AXL Inhibitor, in Lung Cancer Cells With Resistance to EGFR Tyrosine Kinase Inhibitors by MET or AXL Activation

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Background: Although EGFR tyrosine kinase inhibitor (EGFR-TKI) such as gefitinib or erlotinib has shown remarkable response in non-small cell lung cancers (NSCLC) with EGFR mutations, drug resistance eventually develops in the majority of patients, limiting the median response duration to less than one year. Activation of bypass signals such as MET is one of important mechanisms for acquired resistance to EGFR-TKIs.

Material and Method: We investigated antitumor activity of NPS-1034, a newly developed synthetic drug targeting both MET and AXL in established NSCLC and RCC cells with acquired resistance to gefitinib or erlotinib (HCC27/GR and HCC27/ER, respectively) more than 1000 times from HCC2829 harboring deletion mutation on exon 19 of EGFR. Characterization of H820 cells and evaluation of NPS-1034 activity in H820 were also performed.

Result: The resistance of HCC27/GR was mediated by MET activation whereas AXL activation led to resistance in HCC2827. The combination of gefitinib or erlotinib with NPS-1034 synergistically inhibited cell proliferation of both resistance cell lines although there was no effect in either of these drugs. According, suppression of Akt was noted only with treatment of both drugs. NPS-1034 was also effective in subcutaneous xenograft mouse models of HCC2827/GR. Although H820 cell line was previously reported to have T790M and MET amplification, we additionally found that AXL was also activated in this cell line. There were no antitumor effects in EGFR or MET modulation by specific siRNA against EGFR or PHA665752, a small molecule MET inhibitor, combined with erlotinib. However, the addition of NPS-1034 to erlotinib could control H820 cells suggesting AXL would be the main signal to maintain resistance.

Conclusion: Our study showed the efficacy of NPS-1034 in NSCLC cells with resistance to EGFR-TKIs through bypass signals of MET or AXL.

970 Salinomycin Induces Growth Arrest, Apoptosis and Senescence Through HDAC2 Inhibition and Upregulation of Histone Hyperacetylation in the H820 Cancer Cell Line

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Background: Salinomycin is a monacanaric polyether antibiotic, isolated from Streptomyces albai, widely used as an antibiotic to prevent anticoagulosis in poultry. The purpose of this study was to assess whether Salinomycin would induce cell growth arrest and apoptosis in the Estrogen Receptor (ER) positive MCF-7 and T47D and ER negative MDA-MB231 breast cancer cells.

Methods: Antiproliferative/pro-apoptotic effects of Salinomycin were measured with cell tile-Glo (Promega). Cell cycle distribution and apoptosis were determined by flow cytometry using BD FACS cantoll flow cytometer (BD Biosciences). The expressions of proteins of interest were measured by Western blotting and immunofluorescence. Senescence was assayed by the SA-beta-Galactosidase assay. The effect of salinomycin on colony growth was measured using soft agar colony assay.

Results: We demonstrated that treatment of the ER negative MDA-MB231 breast cancer cell line with lower concentration 2.5-10nM led to G1 cell cycle arrest, growth inhibition and senescence, while treatment with high concentration of salinomycin (25 and 50 nM) resulted in apoptosis. Accumulation of cells at G1 correlated with decrease in cyclin D1 and an increase in the cyclin kinase inhibitor, p21, Bcl2 and survivin protein level. Activation of p-h2AX, a marker for double strand break, was also detected 6h after salinomycin treatment. Immunofluorescence staining revealed that salinomycin induced hyperacetylation of H3K9 of the cell cycle arrest. Interestingly, we show that salinomycin treatment of the MDA 231 induced a dose dependent decrease in the level of the Histone deacetylase 2 (HDAC2) protein and increase in the level of acetylated Histone H3 and H4 revealed by Western blotting and immunofluorescence. Furthermore, we show that, in combination assay, salinomycin enhances the antiproliferative effects of Hydroxymatoxin on the ER(+) MCF-7 and frondoside A on the ER (-) MDA-MB231 breast cancer cell line.

Conclusion: Salinomycin possesses potent anti-breast cancer activity that correlated with the down regulation of HDAC2, induction of Histone hyperacetylation and regulation of proteins important for keeping cell cycle arrest, growth inhibition, and apoptosis.

971 Origanum Majusor Extract Induces Apoptosis and Suppresses Migration and Invasion of MDA-MB-231 Human Breast Cancer Cell Line Through Inactivation of the NFkB Pathway

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Background: Breast cancer is the most prevalent malignancy in women in many countries worldwide. Currently, there is no effective therapy for malignant estrogen-independent breast cancer. Treatment of cancers by plant extracts and phytochemicals has become a flourishing field of research over the past decade. Origanum majorana L. (O.M) is aromatic plant native to Mediterranean. Recent studies reveal that OM has several biological activities including anti-microbial and anti-oxidant activities. However, the anti-cancer activity in human has not been reported to date.
Methods: Cell proliferation assay was performed using cell-liter-Glo (Promega) to measure cell viability. Apoptotic cells were determined by flow cytometry using 8-d FACS cantart flow cytometer (BD Biosciences). The invasive and migration behaviors of MDA-MB-231 cells were tested using the Matrigel chamber (BD, Bioscience) and wound healing migration assays, respectively. The expressions of proteins of interest were measured by Western blotting and immunofluorescence. Cytotoxic effect of Origanum extract on colony size was measured using soft agar colony assay.

Results: Here we show that Origanum extract, possesses an anti-proliferative and pro-apoptotic activities against various cancer cell lines in vitro including the Estrogen Receptor negative breast cancer cell line MDA-MB-231. Apoptosis in MDA-MB-231 was confirmed by Annexin V propidium iodide staining, nuclear morphology and caspase 3/7 activation. Protein levels of the antiapoptotic proteins P21 and survivin decreased after 24 h treatment. Origanum extract was also able to reduce dramatically the size of the MDA-MB-231 established colonies in soft agar colony assay. Moreover we show that treatment with non-cytotoxic concentrations dramatically inhibited the migration and the invasiveness abilities of the MDA-MB-231 cells. Treatment of the MDA-MB-231 with the extract induced double strand DNA damage revealed by an increase in the phospho-H2AX protein level. Furthermore, we show that Origanum extract exerts its pro-apoptotic effect through inhibition of the NFκB pathway.

Conclusion: Origanum majorana extract potently inhibited various tumor cell growths in vitro. The anti-cancer activity of this plant extract is mediated by induction of apoptosis and inhibition of the NFκB pathway. Hence, Origanum extract could lead to the development of potent anti-cancer agent for the treatment of diverse cancers.

[972] A Functional Pharmacogenetic Screen in Non-small Cell Lung Adenocarcinoma

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Background: Lung cancer is the leading cause of cancer-related death in western countries, mainly due to late diagnosis. In the past decade, deep sequencing projects have shed light on the different subtypes of lung cancer based on its landscape of somatic mutations. This has supported the evidence that the genetic background of tumors is highly diverse and complex and this determines the patient response to the treatment.

Recently, the first drugs targeting lung cancer specific driven mutations have opened a new era for personalized medicine. Still, most of the patients develop drug resistance within the first year of treatment. Thus, it is crucial to develop new approaches and identify more effective drugs.

One way to achieve this is the concept of synthetic lethality (SLL). Two genes display a synthetic lethal interaction when loss of function of one or the other has a lethal effect as proliferation, while loss of both results in cell death. In the context of cancer, we specifically look for gene-drug interactions that would compromise cell viability in cancer cells while leaving their healthy counterparts unaffected.

Material and Methods: We focus on non-small cell lung adenocarcinoma. In order to mimic the molecular heterogeneity of patients we established a genetically tractable cell model. To do so, we generated 100 isogenic cell lines, each one carrying a driven mutation that triggers the tumorigenesis (i.e. EGFR, KRAS, EML4-ALK . . . ) and a second mutation frequently found in lung adenocarcinoma. These cells are screened in a multiplex assay against a panel of drugs, currently used in clinics or in clinical trials.

Each cell line is tagged with a unique short DNA sequence called barcode, allowing their identification in a mixed population of cells. This method has recently been employed successfully to identify novel synthetic lethal interactions and mechanisms of resistance in breast cancer cells (Mueller et al., Nat. Chem. Biol. 2011). SLL interactions can thus be determined by measuring the abundance of the barcodes, which is indicative for cell viability.

Results and Discussion: We have performed a first screen between our lung cancer-relevant isogenic cell lines and a panel of drugs including kinase inhibitors and classical chemotherapeutics. Currently, we are validating the most significant hits.

Conclusions: Our system provides a systematic and robust method to investigate genotype-specific vulnerabilities upon drug treatment in a high-throughput manner.

[973] Metabolic Profile of Cisplatin-resistant Patient-Related Tumor Cell Lines

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Introduction: Resistance is the main limitation to the clinical usefulness of cisplatin as an anticancer drug and considerable efforts have been undertaken to solve this problem throughout the past three decades. Mitochondrial DNA instability and ROS-induced oncogenic transformation are well documented in cancer, and metabolic strategy to protect newly synthesized DNA against oxidative damage and cytotoxic drugs have been recently proposed as cell adaptive mechanisms. The aim of this study was to investigate the metabolomic profile and the mitochondrial influence on cisplatin-resistance.

Material and Methods: Human ovarian cancer cells (2008 cell wild type and C13 cisplatin-resistant) were used and parent cells derived after depletion of the mitochondrial DNA (rho0-clones). We tested the viability and growth was measured by MTT and trypan blue exclusion test; the transmembrane mitochondrial potential (ΔΨm) and ROS generation by flow cytometry; the oxygen consumption by Clark-type oxygen electrode; the metabolomic pattern by 1H-NMR spectra; lipid droplets by Nile red staining and confocal microscopy.

Results and Conclusion: As regards cytotoxicity of cisplatin results showed IC50 value significantly higher in 2008-rho0 than in 2008 cells (3.56 μM and 0.72 μM) and similar to that in resistant cells; by contrast IC50 did not vary between C13-rho0 and C13 cells (IC50 5.49 μM and 6.49 μM). In comparison with 2008 cells, the C13 cells were more glucose-dependent for survival, they showed lower basal oxygen consumption, lower basal (ΔΨm) and lower sensitivity to rotenone and oligomycin mitochondrial inhibitors. 1H-NMR spectroscopy demonstrated higher basal content of intracellular GSH and mobile lipids (MIs) in C13 cells, as compared to 2008 cells; Nite red staining confirmed the higher lipid content as cytoplasmic droplets in C13 cells. The results support the hypothesis of a ‘metabolic reprogramming’ of ovarian cancer cells to a lipogenic phenotype with consistent changes in glycolytic rate and mitochondrial respiratory pattern. Therefore novel metabolic strategy might be advantageous for cancer cells to escape apoptosis induced by cisplatin. This prompts to investigate further the metabolic reprogramming as a critical goal to identify pharmacological targets to circumvent the cisplatin resistance.

[974] Selective Inhibitors of Nuclear Export (SINE) CRM1 (XPO1)

Antagonists Activate Multiple Tumor Suppressor Pathways and Kill Prostate Cancer (PrCa) Cells

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Background: CRM1 (Xpo1) is the sole export mediating transport of multiple tumor suppressor proteins (TSP) including p53, pRB, FOXO, APC and P16 out of the nucleus, abrogating their function. Many tumor types show overexpression of CRM1, thus extinguishing TSP function. CRM1 inhibition forces nuclear accumulation of TSPs inducing apoptosis in cancer cells.

Methods: MSKCC PrCa gene database was used for mRNA analyses. Inhibition of CRM1 mediated NE was determined by IHC. MTT assays were used to determine cytotoxicity across diverse PrCa cell lines: LAPC-4 (p53wt, AR+, low Akt/mTOR); LnCaP (p53wt, AR+, high Akt/mTOR); LnCaP-C81 and LnCaP-C4-28 (p53wt, AR+, androgen independent, high Akt/mTOR); 22r1v (p53wt, AR+, androgen independent, low Akt/mTOR); PC3 (p53-del, AR-, high Akt/mTOR); DU145 (p53mut, AR+, low Akt/mTOR); SINE are CRM1 inhibitors, across PrCa cell lines. SINE are 1H-NMR spectroscopy demonstrated higher basal content of intracellular GSH and mobile lipids (MIs) in C13 cells, as compared to 2008 cells; Nite red staining confirmed the higher lipid content as cytoplasmic droplets in C13 cells. The results support the hypothesis of a ‘metabolic reprogramming’ of ovarian cancer cells to a lipogenic phenotype with consistent changes in glycolytic rate and mitochondrial respiratory pattern. Therefore novel metabolic strategy might be advantageous for cancer cells to escape apoptosis induced by cisplatin. This prompts to investigate further the metabolic reprogramming as a critical goal to identify pharmacological targets to circumvent the cisplatin resistance.

Results: Asregards cytotoxicity of cisplatin results showed IC50 value significantly higher in 2008-rho0 than in 2008 cells (3.56 μM and 0.72 μM) and similar to that in resistant cells; by contrast IC50 did not vary between C13-rho0 and C13 cells (IC50 5.49 μM and 6.49 μM). In comparison with 2008 cells, the C13 cells were more glucose-dependent for survival, they showed lower basal oxygen consumption, lower basal (ΔΨm) and lower sensitivity to rotenone and oligomycin mitochondrial inhibitors. 1H-NMR spectroscopy demonstrated higher basal content of intracellular GSH and mobile lipids (MIs) in C13 cells, as compared to 2008 cells; Nite red staining confirmed the higher lipid content as cytoplasmic droplets in C13 cells. The results support the hypothesis of a ‘metabolic reprogramming’ of ovarian cancer cells to a lipogenic phenotype with consistent changes in glycolytic rate and mitochondrial respiratory pattern. Therefore novel metabolic strategy might be advantageous for cancer cells to escape apoptosis induced by cisplatin. This prompts to investigate further the metabolic reprogramming as a critical goal to identify pharmacological targets to circumvent the cisplatin resistance.
oral SINE KPT-251 showed dose-dependent inhibition of tumor growth and synergy with doxorubicin.

**Conclusion:** These data show that PrCa shows coordinate NPC regulation, and that selective blockade of CRM1 dependent NE represents a completely novel, neoplasia-selective and well-tolerated target for use as single agent or in combination with chemotherapy for PrCa.

**975** COMT Genetic Variation May Influence Opioid Dosing 

Requirements in the Treatment of Cancer-related Pain

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**Background:** Opioid analgesics are the mainstay treatment for moderate to severe cancer-related pain. However, clinical studies suggest that genetic variability may result in significant differences in response to opioids. The μ-opioid receptor (OPRM1) is the primary site of action for opioids. The polymorphism A118G is relatively frequent in Caucasians and causes an amino acid change from asparagine to aspartatic acid. This polymorphism seems to influence opioids action, with homozygous for A allele requiring lower doses of opioids. Catechol-O-methyltransferase (COMT) is involved in the metabolism of catecholamines, which have a role in the nociception mechanism. The functional polymorphism Val158Met codes the substitution of valine (Val) by methionine (Met). Individuals with the Met/Met genotype have the lowest activity of COMT and have been related to increased pain sensitivity and lower μ-opioid system activation. Polymorphisms in multidrug resistance protein (MDR1) can have pharmacologic consequences after opioids administration. Two of the most frequent polymorphisms are C3435T and C1236T. Homozygous individuals for T allele of the C3435T have lower mRNA expression. C1236T was found to be in linkage disequilibrium with C3435T and was also related to different opioid doses, higher in TT individuals. Our purpose was to investigate the effects of these polymorphisms on several pain-related parameters in Caucasian cancer patients.

**Material and Methods:** DNA samples from 30 cancer patients were genotyped for polymorphisms in OPRM1 (rs1799971), COMT (rs4680), and MDR1 (rs1128503, rs1045642) with Real-time PCR. Daily doses were re-expressed as oral morphine equivalents. We examined the relation between the polymorphisms and opioid dose, pain intensity, performance status, adverse effects, age, gender, body mass index (BMI) and breakthrough pain. Results: Total morphine consumption was related to the polymorphism Val158Met in COMT gene, with carriers of Met allele showing to be significantly associated with higher dose of opioids (p = 0.004, Pearson r² test), which was also confirmed by logistic regression to adjust to gender and age (p = 0.013). All the other polymorphisms and parameters revealed no statistically significant association. Conclusion: This preliminary result indicates that genetic variation at COMT enzyme may influence opioid dosing requirements in the treatment of cancer-related pain. Acknowledgments: FCT grant SFRH/BDE/62779/2009, C-I-POP 11/2008.

**976** Effects of Dual PI3K and mTOR Inhibition on Incidence and Local Growth of Prostate Cancer Bone Metastases

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**Introduction:** Although phosphoinositide 3-kinase PI3K activation is associated to prostate cancer (PCa) progression, the contribution of mTOR and its interaction partners toward regulating PCa progression and metastasis remains poorly understood. The main aims of this report are: (i) to verify if the inhibition of PI3K/Akt/mTOR pathway by using the dual PI3K/mTOR kinase inhibitor, X480, is able to modulate cell proliferation and apoptosis in prostate cancer cells; (ii) to compare the effects of X480 with the mTOR inhibitor, X414; (iii) to verify the effects of these drugs on the production of proteases involved in cell migration and metastasis; and (iv) the final endpoint of this study is to examine the role PI3K/AKT/mTOR inhibition in the incidence of bone metastases as well as its role on the growth of tumor cells in the bone microenvironment.

We analyzed the cytotoxic effects and biochemical arrangement induced by these compounds on prostate cancer cell line cells and a series of 6 prostate cancer cell lines and 11 prostate cancer cell derivatives. In vivo, male nude mice were injected with PC3cells by intracardiac (metastatic model) and intratibial (local growth) method. Dual inhibition of PI3K/mTOR signaling, using X480 attenuated cell proliferation and induced apoptosis in prostate cancer cells. The effects were more marked in prostate cancer cell line cells, a Pten loss and Akt activation (by increased EGFR/Her2 activity or PIKCA mutation). mTOR inhibition by X414 was also able to reduce cell proliferation and to induce apoptosis. Cell migration and invasion experiments indicated that the inhibition of the PI3K/mTOR pathway was associated with marked reduction of metastatic potential. The analyses of in vivo experiments revealed a significant reduction of metastases incidence and tumor burden after treatment with X480 (p < 0.05). Evaluation of tumor growth in bones after intratibial injection revealed a significant reduction both after X480 and X414 treatments. Tibiae obtained 35 days after intratibial injection of nude mice were analyzed by Xray and microCT and scored as: score 0 (absence of tumor growth after xRay and microCT analyses), score 1 (osteoelastic lesions visible only to microCT analyses and <5mm²), score 2 (lesions visible only microCT and <5mm²), score 3 (lesions visible to xRay without cortical impairment), score 4 (Xray visible lesions with cortical impairment with/without fractures) and score 5 (extended osteolysis with extra-tibial growth). X480 was able to significantly reduce tumor burden (4/20 tibiae with score >3) relative to those observed in untreated animals (12/20 tibiae with score >3, p < 0.05). Although mTOR selective inhibition by X414 was able to reduce tumor burden (7/20 tibiae with score >3), this was not significant. However, histo-morphometric analyses revealed that X414-treated animals showed significant reductions of tumor mass (p < 0.05). These findings provide the rationale for including PI3K/Akt/mTOR kinase inhibitors as part of the therapeutic regimen for PCa patients and suggest that dual inhibition of PI3K and mTOR may more effectively inhibit metastasis and tumor host interaction in the bone microenvironment than inhibition of mTOR alone.

**977** Dehydroxymethylepoxyquinomicin, a Novel Inhibitor of NF-κB Efficiently Suppresses Invasion and Radiosensitizes Glioblastoma Cells

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**Introduction:** Glioblastoma (GBM) is the most aggressive primary brain tumor. Despite the improvements in neurosurgery, radiation management, and the advent of Temozolomide (TMZ), its treatment is hampered by its inherent chemoresistance and its ability to invade/infiltrate surrounding tissues. The transcription factor NF-κB is constitutively activated in most tumors, including GBM. By virtue of its ability to regulate the expression of genes involved in apoptosis, differentiation, chemo-resistance, migration and survival, it constitutes a point of convergence of many oncogenic pathways. Recently, Dehydroxymethylepoxyquinomicin (DHMEQ) has shown to be a potent NF-κB inhibitor with anti-tumor and chemosensitizing properties.

**Material and Method:** In the present study the effects of NF-κB inhibition by DHMEQ (alone or combined ionizing radiation) were evaluated in 6 adult (U251, U343 MG-a, U138 MG, U87 MG T86G and LN319) and 1 pediatric (SF-188) glioblastoma cell lines by means of proliferation (XTT assay), viability (Trypan blue exclusion), cell cycle dynamics, clonogenic capacity, apoptosis (Nacvihue 488 caspase-3 kit), necrosis (propidium iodate staining), migration (wound healing scratch) and invasion on matrigel assays. Cells were treated with different concentrations (2.5, 5, 10 and 20 μg/ml) for 24, 48 and 72 hours. All experiments were performed on triplicates and results compared by two-way ANOVA followed by Bonferroni test.

**Results:** Our results showed that DHMEQ treatment resulted in impaired cell proliferation with G2/M arrest in dose and time-dependent manner when compared with control for all cell lines. Cell viability and clonogenicity were also significantly diminished with increasing dose of DHMEQ and necrotic cells. Cell migration and invasion were inhibited accordingly. When combined with ionizing radiation, 24h pretreatment of cells with 10 μg/ml dramatically sensitized cells to ionizing radiation at 2, 4 and 8 Gy doses. Conclusion: Although efforts are made in order to improve GBM survival, most drugs developed so far have shown disappointing results in clinical trials. Our results show the anti-tumor effect of DHMEQ suggesting being a good candidate for a new chemotherapeutic agent against pediatric gliomas, to be further exploited on new models. Financial support: FAPESP (proc. 2010/16652-9 and 2010/50635-4).
Effects of 1D11 an Antibody Against Transforming Growth Factor Beta (TGF-β) on Incidence and Local Growth of Prostate Cancer Bone Metastases
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Background: Transforming growth factor beta (TGF-β) plays an important role in prostate cancer (PCa) development and this growth factor has been implicated in the pathogenesis of PCa-derived bone metastases.

Material and Methods: The main aims of this report are to verify if the inhibition of TGFβ pathway, by using the pan-neutralizing anti-TGF-β antibody (1D11) or its murine form 13C4, could control PCa bone metastases and inhibit growth of tumor cells in the bone microenvironment. 1D11 is a murine monoclonal antibody against all three TGFβ isoforms and has been used as a therapeutic agent for a fully human antibody (GC100B) currently in clinical development. We analyzed the effect of 1D11 on the growth of C4-2B and PC-3 bone derived PCa cells in vitro by measuring radiolabeled thymidine incorporation into DNA as well as the biochemical, histological, and histomorphometric analysis of biopsies obtained from nude mice injected with intratibial injection of nude mice. Tibiae obtained 35 days after injection of nude mice were analyzed by X-ray and microCT and scored as: score 0 (absence of tumor growth after X-ray and microCT analyses), score 1 (osteolytic lesions visible only to microCT analyses and <5mm²), score 3 (lesions visible to X-ray without cortical impairment), score 4 (X-ray visible lesions with cortical impairment with/without fractures) and score >5 (extended osteolysis with extra-tibial growth). 1D11 was able to significantly reduce tumor burden (6/20 tibiae with score 3) relative to those observed in untreated animals (14/20 tibiae with score >3, p < 0.05).

Conclusions: In summary, we report for the first time that TGF-β neutralization with a monoclonal antibody (1D11) can control PCa bone metastases and it may be a useful therapeutic approach in men with advanced PCa.

Development of a Trans-kingdom Expression System in Cancer Therapy
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Background: Several bacterial species such as Clostridium Sp, Escherichia coli and Salmonella sp. effectively colonize solid tumors and used as anticancer therapeutics. The naturally occurring antitumor activity of Salmonella typhimurium 7207 strain can be further enhanced by genetic manipulations, making it a promising system for targeted delivery and expression of therapeutic molecules in solid tumors.

Methodology and Principal Findings: The engineered strain, termed ST1, was generated by the Lambda-Red recombineering method. First, T7 RNP gene was inserted into the chromosome to direct the transcription of small RNA or exogenous gene. The second step is to replace an essential gene by a pore-forming factor which encodes a bacterial factor needed for maximal transfer of therapeutic factors in vivo. Then we cloned back this essential gene with anaerobic control. The final step is to delete another essential gene to achieve stable plasmid maintenance in vivo. In breast tumor bearing nude mice, ST1 could target the hypoxic and necrotic regions of solid tumor with no obvious side effect. The anticancer effect of ST1 can be exerted in different approaches: (a) Bacterial protein delivery: as a protein delivery vehicle, ST1 encoding the extracellular domain of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, amino acids 114–281) expresses soluble TRAIL(sTRAIL) directly in extracellular environment or inside tumor and induce apoptosis of these cells. ST1 (sTRAIL) mediated delivery and stability of exogenous proteins may promote their successful delivery to target cancer cells. (b) Bactofection: ST1 has been used to transfer therapeutic gene DT through a trans-kingdom dual expression system in MDA-MB-231 xenograft tumor model. After intravenous injection of ST1 carrying toxic gene, vector-derived mRNA and toxin proteins were detected inside tumors. The significant shrinkages of breast tumors were with no gross sign of toxicity. (c) Trans-kingdom RNA interference (RNAi): ST1 carrying a trans-kingdom RNAi system is able to transfer RNAi effector molecules between bacteria and mammals in vivo. ST1 could deliver short-hairpin RNAs (shRNAs) and transduce plasmid-encoded short hairpin RNAs (shRNAs) to induce RNAi against the breast cancer oncogene catenin(β)-1, into the cytoplasm of the cancer cells. Systematic administration of ST1 (TRANS-shCat) induced a potent and specific silencing of catenin(β)-1 in the tumor and retarded its development.

Conclusion: ST1 is an innovative cancer therapy system. The successful development of breast cancer growth and the absence of detectable cytotoxicity suggest the potential that engineered tumor-targeting Salmonella ST1 can be exploited as a targeting vehicle of therapeutic molecules and as a therapeutic ‘factory’ for use in an innovative cancer therapy.

Genetic Risk Factors of Neurotoxicity During Chemotherapy
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Background: Several SNPs were determined to be associated with neurotoxicity during chemotherapy. The main aims of this report are: to verify if these genetic variations on the side effects of chemotherapy, but the combination of genotypes is strongly associated with neurotoxicity.

Conclusion: Our results indicate that genetic variations in genes involved in drug resistance may have high impact on the acute CNS toxicity of grade II or above according to the Common Terminology Criteria for Adverse Events v3.0. Logistic regression adjusted for potential confounders was performed using SPSS 19.1 software.

Biological Effect of Resveratrol Metabolites on Intestinal Epithelial Cancer Cell Growth
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Background: Resveratrol (3,4′,5′-trihydroxy-trans-stilbene, RV) is an active polyphenol with numerous physiological properties including antitumor activity, especially in colon cancer. Resveratrol is metabolised in enterocyte, and to a lesser extent in colonocyte, and conjugated to glucuronidated or sulfated forms that are exported to target organs and after exert their effect are eliminated with urine and stool.

Material and Methods: On the basis of RV metabolism, we select three metabolites: 3-sulfate RV, 3-glucuronide RV, 4′-glucuronide RV to study their effects in cell growth, DNA synthesis, cell cycle and apoptosis in human adenocarcinoma cell (Caco-2) cultures.

Results: Our results show that RV metabolites have an antioxidant activity similar to that of RV. Moreover, all metabolites inhibited cell growth in a concentration-dependent manner as well as [3H] thymidine incorporation. Furthermore, we observed an increase in the percentage of cells in S phase induced by RV metabolite treatments as well as the induction of apoptosis.

Conclusions: On the basis of our results we propose that RV metabolites represent an active contribution to the successful delivery to target cancer cells and to the health benefit attributed previously only to RV. These metabolites are a potential target for more research into the prevention and treatment of colorectal cancer.

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**Updated Management Options in Chronic Myeloid Leukemia**

**Rapid and Simple Method for Detecting EGFR Gene Mutation**

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*Poster Sessions*

**Background:** In chronic myeloid leukemia (CML) conventional chemotherapy (CChT) does not markedly reduce Ph-positive cells, therefore transformation to acute phase occurs. Tyrosine kinase inhibitors (TKIs) have changed the landscape of the treatment of CML. Gleevec® International Patient Assistance Program (GIPAP) is the first global direct-to-patient access program, providing imatinib mesylate (IM) for patients (pts) with CML and CD117 positive unresectable and/or metastatic gastrointestinal stromal tumors. The objectives of the study were to evaluate the results of implementation of GIPAP in Moldova and to perform the comparative analysis of responses to CChT and TKIs in different phases of CML.

**Material and Methods:** 125 CML pts had been followed up at the Institute of Oncology during the period of 2008–2010. 74 (59.2%) pts were managed with IM within the framework of GIPAP. IM was used as a front-line therapy in 11 (14.9%) cases, and to perform the comparative analysis of responses to CChT and TKIs in different phases of CML.

**Results:** Of pts receiving IM via GIPAP 65 (87.8%) were in chronic phase, and to perform the comparative analysis of responses to CChT and TKIs in different phases of CML.

**Conclusion:** The purpose of this study was to comparatively investigate in vitro and in vivo anti-cancer properties as well as adverse effects of two methyl-substituted enantiomerically pure oxaliplatin analogues (KP1537, KP1691) and to evaluate the impact of stereoisomerism.

**Methods:** Cytotoxic and antiproliferative effects were tested against selected human cancer cell lines. Platinum accumulation and DNA binding was determined by ICP-MS. For in vivo experiments, cells were transplanted i.p. (leukemic) or s.c. (solid tumor) into immuno-competent or -deficient mice. For in vitro studies mice were analyzed for hypersensitivity towards heat, cold, and mechanical stress.

**Results:** Despite widely sharing the mode of action with oxaliplatin, several additional positive features were found for the novel analogs. Thus, KP1537 and KP1691 activities were less vulnerable by platinum drug resistance mechanisms and p53 mutations. While oxaliplatin was active solely in an immuno-proficient background in vivo, a phenomenon known as immunogenic cell death, anticancer activity of KP1537 and KP1691 exhibited reduced dependency on the immune system. In mice bearing L1210 leukemia, KP1537 but not KP1691 was significantly superior to oxaliplatin in promoting overall survival. Furthermore, distinctly attenuated adverse effects including weight loss and cold hyperalgesia, could be detected after KP1537 treatment in comparison to oxaliplatin. Interestingly, the reduced hypersensitivity towards cold was accompanied by reduced platinum levels in the sciatic nerve. Generally, substitution and stereoisomerism of one methyl group has a considerable and in some aspects even contradictory impact on drug accumulation both in vitro and in vivo. KP1537 led to higher total cellular and DNA-platinum levels as KP1691 or oxaliplatin in vitro. However, in wash-out experiments no cell-associated platinum was lost during the drug-free-post-incubation in case of oxaliplatin and KP1691, while the KP1537-associated cellular reduction was by about 50%. In contrast, tumor platinum levels after KP1537 treatment were not reduced over time.

**Conclusion:** Summarizing, methyl-substituted oxaliplatin analogues harbor improved therapeutic characteristics including significantly reduced adverse effects. Hence, they might be promising metal-based anticancer drug candidates for further (pre)clinical evaluation.

**The Role of Bystander Effects in the Anti-tumour Activity of the Hypoxia-activated Prodrug PR-104a**

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**Background:** The phosphate ester pre-prodrug PR-104, currently in clinical trial, is designed to exploit tumour hypoxia by releasing the alcohol PR-104A, which is activated at low pH by redox cycling of pyridine nucleotides (NADPH). PR-104A diffuses out of the cells in which it is formed, but it is not known whether this ‘bystander effect’ provides killing of surrounding aerobic tumour cells. We approached this question by developing a spatially resolved assay based on measured plasma levels of PR-104A, H and M and parameters defining the transport in xenografts and cytotoxicity in tumor cells (WT) and isoelectric cells overexpressing a soluble form of POR (sPOR).

**Cellular uptake, metabolism and potency of PR-104A:** H and M were assessed in single cell suspensions (SCS) and diffusion coefficients were measured using multicellular layers (MCLs). Cell kill and hypoxic fraction in tumour xenografts were measured using clonogenic assay and the hypoxia probe EFS, respectively.

**Results:** PR-104A transport through MCLs was impaired by metabolic consumption under hypoxia, but was accompanied by efflux of reduced metabolites on either side of the MCL. The diffusion coefficients of PR-104H and M were similar to that of PR-104A (1.4 x 10⁻⁷ cm²/s). SCS experiments showed cellular uptake of PR-104A and partial retention of metabolites.

**Conclusion:** Cell kill was linearly dependent on intracellular exposure of PR-104H and M. PR-104A showed 7-fold higher levels in sPOR than in WT cells. At the MTD (1330 µmol/kg PR-104) cell kill was 3.4-fold higher in sPOR than in WT tumours (p < 0.001). Even in WT the proportion of cells killed (84.6 ± 5.5%) greatly exceeded the hypoxic fraction (21.2 ± 0.8%). The SR-PKPD model identified two main mechanisms for killing of aerobic cells: a bystander effect resulting from intra-tumour hypoxic activation and killing of cells near blood vessels by circulating PR-104H and M.

**Conclusions:** The in vitro, in vivo and in silico models suggest that PR-104A is able to elicit a bystander effect whose magnitude is critical dependent on the extent of hypoxic reductive activation. This demonstrates a need to identify the PR-104A activating reductases and to screen their tumour expression in patients.
samples and 2 archival cytological smears from 42 lung adenocarcinoma patients were evaluated. EGFR-activating mutations, that is, L858R (exon 21) mutation, exon 19 deletion, and EGFR-TKI-resistant T790M (exon 20) mutation, were analyzed by mutation-based polymerase chain reaction (PCR) performed using a quenching probe (MBP-QP) and TM analysis using the i-densy system (ARKRAY Inc.). The cytological samples were analyzed in sediments of cells without DNA extraction. Paraffin-embedded biopsy and archival cytological samples were analyzed in extracted DNA. The results for the MBP-QP and TM method were compared with those for the PCR clamp and DNA direct sequencing methods.

**Results:** The L858R mutation, exon 19 deletion, and T790M mutation were detected in 6, 6, and 2 cases, respectively. Of the patients with L858R mutation or exon 19 deletion, 8 were treated with EGFR-TKIs. Six patients were responsive to this treatment. However, 2 patients were resistant to EGFR-TKI treatment, and the T790M mutation was detected in addition to L858R mutation or exon 19 deletion in these patients. These EGFR mutations were also detected by the PCR clamp method and DNA direct sequencing.

**Conclusions:** The MBP-QP and TM method used in the current study is a rapid and simple method for simultaneously detecting EGFR-activating and gatekeeper mutations. It is expected to be useful for companion diagnosis for determining EGFR-TKI treatment in lung adenocarcinoma.

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**[Detection of Drug-target Proteins on Tumor-derived Exosomes](#)** by [ELISA Using Anti-CD81 Antibodies](#)

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**Introduction:** Detection and measurement of drug-target or biomarker proteins in tumor tissues are important processes for personalized cancer chemotherapy. However, current immuno-histochemical or cytogenetic analyses of biopsy specimens are highly invasive for pre-therapeutic diagnosis and are often difficult to apply to lung cancer. In this study, we established a simple method to detect membrane proteins such as epidermal growth factor receptor (EGFR) on exosomes secreted by tumor cells and evaluated its usefulness as a minimally invasive diagnosis.

**Material and Methods:** Cell culture: Human lung cancer cell lines (HARA, HARA-B and A549) were maintained in RPMI-1640 with 10% fetal bovine serum and cultured for 72 h in the serum-free culture medium to purify exosomes. Exosome preparation: The culture medium were sequentially centrifuged at 300 g for 5 min and 16,000 g for 20 min to eliminate cells and debris, followed by filtration through a 0.22 μm pore nitrocellulose membrane. Then, exosomes were precipitated by ultracentrifugation at 100,000 g for 72 h in the serum-free culture medium to purify exosomes. Exosome detection by sandwich ELISA: Mouse anti-human CD81 antibody was immobilized on assay plate to capture exosomes specifically. For target protein detection, goat anti-EGFR antibody and anti-goat IgG were used. Blood plasma: As a model of patients’ plasma, peripheral blood of BALB/c nu/nu mice inoculated with HARA-B cells was collected and EGFR on exosomes was measured with the ELISA.

**Results and Discussion:** Anti-CD81 antibody was used for capturing the exosomes because CD81 presented universally in each exosome membrane of normal and lung cancer cells. An ELISA system that captures exosomes using an anti-CD81 antibody, successfully detected exosomal EGFR, a typical target of molecular targeting drugs, from EGFR-positive tumor cell exosomes and blood plasma of tumor-bearing mice. Results of this study suggest that this novel method for exosomal protein measurement could be useful for in vitro diagnosis, especially for minimally invasive companion diagnosis in personalized cancer chemotherapy.

**Conclusion:** The amount of EGFR expression in plasma exosomes was quantified in order to apply to the diagnosis for lung cancer. The quantification of EGFR in plasma should be utilized for the ‘companion diagnostics’, which is associated with the effectiveness of anticancer drugs such as Cetuximab to detect the EGFR level in plasma derived from lung cancer patients in the near future.

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**Molecular Mechanisms of Sorafenib-induced Apoptosis in Cancer Cells**

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**Introduction:** The RAS/MEK/ERK pathway is a key oncogenic pathway involved in human cancer. Point mutations in RAS genes resulting in constitutive activation of RAS proteins have been identified in 30% of the cancers. However, RAS activation results in an overactivation of signaling pathways thereby inducing cell proliferation, invasion and apoptosis inhibition. We investigated the Raf kinase inhibitor Sorafenib and the MEK inhibitor U0126 in human embryonal kidney 293 cells transformed by RAS activation. It was expected to be useful for companion diagnosis for the same pathway; yet, treatment with Sorafenib induces apoptosis while U0126 does not. A time-resolved analysis of this effect by protein and mRNA arrays, revealed an involvement of DDIT4 (also known as Redd1) and mTOR signaling in the differential action of Sorafenib and U0126. The present study aims to examine whether that observation also applies to human cancer cells and to get a better understanding of the molecular mechanisms behind it.

**Materials and Methods:** We treated six different renal carcinoma cell lines with Sorafenib or U0126 by daily cleavage-3 measurement via flow cytometry. In addition, expression of DDIT4 was analyzed using Real-Time-PCR. To further investigate the link between DDIT4 and apoptosis we performed a siRNA-mediated knock-down of DDIT4 as well. The activation of mTOR and associated components of that signaling network were examined using a Bio-Plex assay. The activating transcription factor ATF4 described in literature as a regulator of DDIT4 was also analyzed using Real-Time-PCR.

**Results and Discussion:** Four cell lines induced caspase-3 cleavage and a significant increase of DDIT4 after Sorafenib treatment only. One cell line could not induce DDIT4, one cell line did induce DDIT4, but no cleavage of caspase-3. siRNA-mediated knock-down of DDIT4 inhibited caspase-3 cleavage. A closer examination of the mTOR pathway using the Bio-Plex assay revealed no differences in mTOR inhibition between cells with and without DDIT4-mediated apoptosis. A significant upregulation of ATF4 in combination with an increased RNA level of DDIT4 could only be confirmed in one of the four selected cell lines.

**Conclusion:** These results encourage the hypothesis that the induction of DDIT4 plays an important role for apoptosis induction by Sorafenib in human renal cancer. However, successful induction of apoptosis might be hampered by currently unknown mechanisms interfering with DDIT4-upregulation or caspase-cleavage.

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**Pharmacological Approach of the Role of Transcriptional Activation and Degradation in Acute Promyelocytic Leukemia**

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**Background:** Acute promyelocytic leukemia (APL) is caused by a variety of chromosomal translocations into the retinoic acid receptor-alpha (RARA) gene. The X-RARA fusions tightly associate with corepressor complexes under physiologic concentrations of retinoic acid (RA), leading to transcriptional repression and differentiation block. Only very high RA concentrations allow APL differentiation and clinical remission. It has been proposed that while transcriptional activation likely controls differentiation, may not be the primary pathway for the therapeutic efficacy of retinoic acid in clearing APL. Indeed, only PML-RARA degradation ensures efficient APL eradication. The renewed interest in PML/RARA degradation led us to search molecules that could trigger RARA.

**Material and Method:** To identify new compounds with modulated RARA degradation we have constructed an human GEP-RARA cell line to carry out a cell-based fluorescent assay using libraries of FDA-approved compounds. These tests were used for their ability to oppose RA-induced RARA degradation. Results: The screen blindly identified expected positive controls such as RA itself, rexinoids or proteasome inhibitors. We also identified Etretinate, a synthetic retinoid used in skin disease, as a compound that antagonizes RARA degradation. Unexpectedly, Etretinate, and its active metabolite Acitretin, both induced differentiation in several APL models ex vivo or in vivo, but did not exert significant anti-leukemic effects. Ex vivo, Etretinate or Acitretin treatment did not result in quantitative differences in clonogenic ability of pre-leukemic APL cells. In vivo, Etretinate differentiates APLs without significant loss of clonogenic activity. Importantly Etretinate or Acitretin, in sharp contrast to RA, did not trigger PML/RARA or RARA degradation.

**Conclusion:** Our results uncouple retinoic-induced degradation and transacti-

**RARA.** They establish the role of PML/RARA degradation in retinoic acid response. They pave the way to screens where these two effects of
Acquired Resistance to Aromatase Inhibitors is Paired With...
Development of a Therapeutic Peptide Targeting the Transmembrane Domain of Plexin A1

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Background: Plexin-A1 (PlexA1) is the major signaling co-receptor of Neuropilin-1 (NRP1). Considering the increasing importance of NRPs in the process of tumor growth and dissemination, we are developing novel peptide inhibitors blocking PlexA1 signal transduction in order to inhibit NRPs-associated functions. Our strategy is based on the use of a peptide mimicking the transmembrane domain of PlexA1 (pTM-PlexA1). As previously described with such transmembrane peptide targeting NRPs (Roth et al., 2008, Mol Biol Cell), pTM-PlexA1 disturbs dimerization and signaling leading to significant inhibition of tumor growth.

Material and Methods: We used immunocytochemistry to determine the expression of PlexA1 in human glioma and endothelial cells (U87MG, U118 and HUVEC, HMEC). We performed MTt assay and 3D aggregates migration assay to assess the impact of the peptide on cell proliferation and cell migration respectively. The therapeutic potential of the pTM-PlexA1 has been demonstrated in vivo in a model of subcutaneous tumor (U87MG) grafted in the flank of nude mice. The anti-angiogenic property of the peptide has been evaluated by its ability to block the formation of tube-like structures by HUVEC cells grown on Matrigel.

Results: Our results showed that pTM-PlexA1 inhibits tumor cell proliferation, VEGF-induced tumor cell migration and also significantly reduced the growth of subcutaneous human tumors in vivo. Strikingly, the histological characterization of the subcutaneous tumors revealed that PlexA1 is expressed on-tumor-associated blood vessels thereby suggesting a role in angiogenesis. This expression is also found on endothelial cells (HUVEC and HMEC). Consistently, we found that adding pTM-PlexA1 strongly reduced tube-like structures in the matrigel assay.

Conclusion: Hence, in line with our previous NRPs targeting peptide, blocking the transmembrane domain of Plexin-A1, the use of a transmembrane peptide appears as a valid strategy opening a novel avenue for the development of a new class of membrane receptor inhibitors with clear therapeutic potential.

The Effect of Gossypol in Hepatocellular Carcinoma – Studies with 18F-FDG

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Introduction: Hepatocellular Carcinoma (HCC) overexpresses GLUT1 which is related with a promotion of tumorigenesis. The suppression of GLUT1 expression by siRNA significantly impaired tumorigenicity of HCC cells, is related with a promotion of tumorigenesis. The suppression of GLUT1 expression by siRNA significantly impaired tumorigenicity of HCC cells, therefore, it is an innovative therapeutic target for this aggressive tumor. Positron Emission Tomography (PET) using the radiolabeled glucose 18F-FDG was incubated in a cell suspension with 2*10^6 cells/ml (25 μCi/ml) and then incubated in a cell suspension in vitro. Eppendorf were then centrifuged and radioactivity of cell pellets and supernatants was measured with a well-type gamma counter.

Results: This study demonstrates that the concentrations of gossypol necessary to achieve the IC50 is higher for Huh7 cell line (IC50(gossypol) = 7.5 μM). Interestingly, the cell line more sensitive to this compound is HepG2 (IC50(gossypol) = 2.6 μM). Among the new derivatives displayed a lower toxicity towards human hepatocytes than the parent compound TZD (1-fold when used at 100 μM)

Conclusions: These results shown that gossypol has anti-proliferative effect in vitro. Gossypol is a natural compound that has demonstrated anticancer activity. It appears as a valid strategy opening a novel avenue for the development of a new class of membrane receptor inhibitors with clear therapeutic potential.

New Thiazolidinedione Derivatives With PPARγ/α-Maindependent Activity – High Inhibition of Breast Cancer Cell Proliferation With Low Toxicity Towards Human Hepatocytes

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Background: Breast cancer is the most frequent cancer in women. The development of de novo and acquired resistance to therapies and the absence of targeted therapy for some types of tumours are strong motivations for discovering new therapeutic agents. Thiazolidinediones (TZD) are studied in this context. These PPARγ ligands have antiproliferative effects in vitro and in vivo and they were tested in clinical trials. Nevertheless compounds like troglitazone (TGZ) display hepatotoxicity. More recently, it was shown that in fact, TZD exert their anticancer effects mainly in a PPARγ-independent manner. Therefore, we synthesized new TZD derivatives (Δ2 family) devoid of PPARγ agonist activity. We studied their mechanism of action and their toxicity towards human hepatocytes before performing in vivo experiments on murine models.

Material and Methods: TZD derivatives were obtained by chemical synthesis. We used hormone-dependent (MCF-7) and hormone-independent (MDA-MB-231) breast cancer cell lines as well as primary cultures of human hepatocytes. Proliferation and toxicity were studied with the Cell Titer-Glo assay and the 18F-FDG uptake by the cells respectively. Gene expressions were studied by microarray, RT-PCR, western blotting and immunocytochemistry. RNA interference was used for gene silencing.

Results: The PPARγ-independent activity of Δ2-TZD in breast cancer cells was associated with a very early increase in intracellular calcium (after 2 minutes) followed by ERK1/2 activation (after 5 minutes) and a transient increase in the expression of the transcription factor “Early Growth Response gene 1” (maximum after 3 hours). Endoplasmic reticulum stress and proapoptotic degradation of cyclin D1 and E1c were observed. Δ2-TGZ and TGZ had similar antiproliferative efficiencies: their IC50 were 29.7 and 35.4 μM respectively in MCF7 cells and 16.6 and 15.8 μM in MDA-MB-231 cells. Among the new Δ2-TZD derivatives that we obtained, some of them were more potent than TGZ and Δ2-TGZ with IC50 around 5 μM. Furthermore, these derivatives displayed a lower toxicity towards human hepatocytes than the parent compound TGZ (1-fold when used at 100 μM). Our work contributed to a better understanding of the PPARγ-independent effects of TZD. We were able to obtain derivatives which were more potent to inhibit breast cancer cell proliferation and less toxic towards hepatocytes. This opens the possibility to start in vivo experiments in mouse models of breast cancer.

Treatment of Human Glioma Xenografts With Oncolytic H-1PV – A Comparison of Various Ways of Virus Application

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Material and Methods: Human glioma cell line CGL9 (Oncodesign, France) was implanted into the brain of immunodeficient rats. The tumor growth was further monitored by MR imaging. As the tumor has reached a defined visible volume, the oncolytic virotherapy has been started. Following methods of infection have been used in the experiment: intracranial single injection of virus, intracranial injection performed by convection enhanced delivery (CED), intraventricular injection via osmotic pump. After finishing of experiment, animals have been sacrificed and blood samples or isolated tissues were analyzed for virus distribution, expression of viral proteins and presence/lack of viral toxicity.

Results: After the treatment with H-1PV the volume of implanted human glioma was partially reduced as observed by MR imaging. The survival of treated animals was prolonged in comparison to untreated control group. The animals treated by osmotic pump survived longer than all other experimental groups.

Conclusion: Using the in vivo human glioma system, the collaboration between the DKFZ/INSERM group and ONCODESIGN established the
following results: (I) in the provided system, H-1PV led to partial regression of tumor with prolonged survival of tumor-bearing animals, (II) the method of 5-Fluorouracil was used as the positive control. On day 8, the mice treated with the vehicle (10% DMSO) used for diluting the tested substance. The 5-Fluorouracil was used as the positive control. On day 8, the mice were sacrificed and tumors were excised and weighed. The animal studies of conventional anti-cancer drugs. We hypothesise that increased ERBB expression is associated with decreased sensitivity of MB cell lines to chemotherapy due to up-regulation of survival pathways and that PF-00299804 will improve the efficacy of conventional anti-cancer drugs.

Material and Methods: To evaluate the efficacy of PF-00299804, as a single agent and in combination with conventional chemotherapeutics, we have developed a panel of cell lines representing PB and various paediatric MB subtypes which were examined in vitro and in vivo using orthotopic xenograft. In addition, this study explored the utility of a novel model of MB, the MD2-SmoA1 mouse, which represents the Sonic Hedgehog subgroup of human MB. Immunoblot and immunohistochemical analysis for ERBB1–4 was used to determine ERBB pathway deregulation in our models. 

In Vivo Growth Inhibition of Sarcoma 180 by the Medicinal Plant Kielmeyera Rugosa Choisy (Clusiaceae) 

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Background: The medicinal plant Kielmeyera rugosa Choisy (family Clusiaceae, subfamily: Kielmeyeroideae) is popularly known in Brazil as “pau-santo” and growing in Sergipe and Bahia, northeast Brazil. In a preliminary study, the stem extract of K. rugosa showed cytotoxicity against several tumor cell lines in culture. In this study, the in vivo antitumor activity of stem extract of Kielmeyera rugosa (EK) was demonstrated using experimental model. In addition, systemic toxicological evaluation was also performed.

Material and Methods: The antitumor activity of EK was assessed in mice inoculated with sarcoma 180 cells. Sarcoma 180 cells were injected (2 × 10^6 cells/animal/s.c.) in mice left hind limbs. Young healthy male (n=10) Swiss albino mice (body weight 25-30 g) were used. One day after, the animals were treated intraperitoneally or orally for 7 consecutive days. Negative control was treated with the vehicle (10% DMSO) used for diluting the tested substance. The 5-Fluorouracil was used as the positive control. On day 8, the mice were sacrificed and tumors were excised and weighed. The animal studies of the Federal University of Sergipe approved the experimental protocol (number 56/2010).

Results: The EK showed antitumor activity in both administration routes. Tumor growth inhibition rates were 34.9–40.8% and 25.4–51.8% for the EKR treated by intraperitoneal (50 and 100 mg/kg/day) and oral (100 and 200 mg/kg/day) administration, respectively. The treatment with KRE did not significantly affect the body mass, macroscopy of the organs, or blood leukocytes.

Conclusions: We can conclude that the EKR possesses significant antitumor activity, when administrated by oral or intraperitoneal routes, and has low toxicity.

Assessment of Antitumor Properties of the Essential Oil From the Leaves of Guatteria Friesiana 

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Background: Guatteria friesiana (W.A. Rodrigues) Erkens & Maas (synonym Guatteriopsis friesiana W.A. Rodrigues, family Annonaceae), popularly known as “santo” and growing in Sergipe and Bahia, northeastern Brazil. In a preliminary study, the stem extract of K. friesiana popularly known as “santo” and growing in Sergipe and Bahia, northeastern Brazil. In a preliminary study, the stem extract of K. friesiana showed cytotoxicity against several tumor cell lines in culture. In this study, the in vivo antitumor activity of stem extract of Kielmeyera rugosa (EK) was demonstrated using experimental model. In addition, systemic toxicological evaluation was also performed.

Material and Methods: The antitumor activity of EK was assessed in mice inoculated with sarcoma 180 cells. Sarcoma 180 cells were injected (2 × 10^6 cells/animal/s.c.) in mice left hind limbs. Young healthy male (n=10) Swiss albino mice (body weight 25-30 g) were used. One day after, the animals were treated intraperitoneally or orally for 7 consecutive days. Negative control was treated with the vehicle (10% DMSO) used for diluting the tested substance. The 5-Fluorouracil was used as the positive control. On day 8, the mice were sacrificed and tumors were excised and weighed. The animal studies of the Federal University of Sergipe approved the experimental protocol (number 56/2010).

Results: The EK showed antitumor activity in both administration routes. Tumor growth inhibition rates were 34.9–40.8% and 25.4–51.8% for the EKR treated by intraperitoneal (50 and 100 mg/kg/day) and oral (100 and 200 mg/kg/day) administration, respectively. The treatment with KRE did not significantly affect the body mass, macroscopy of the organs, or blood leukocytes.

Conclusions: We can conclude that the EKR possesses significant antitumor activity, when administrated by oral or intraperitoneal routes, and has low toxicity.

Correlation Between Differences in the Increase in MAPK (ERK1/2) and Cancer:

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Background: Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that, on ligand binding, triggers the RAS-RAF-mitogen-activated protein kinase (MAPK) signaling pathway, which is mainly associated with cell proliferation. In non-small-cell lung carcinoma (NSCLC), known mutations, which are called driver mutations, in EGFR, KRAS, BRAF, and other oncogenes cause continuous activation of tyrosine kinase. In this study, the aim is to clarify the correlation between driver mutations and the activity of MAPK (ERK1/2) in advanced NSCLCs.

Material and Methods: Paraffin-embedded lung biopsy samples were obtained from 110 non-squamous NSCLC patients (study period, 2006–2010). EGFR mutations were analyzed using the PCR clamp method. KRAS codons 12 and 13 and BRAF V600E mutations were assessed by mutation-based PCR performed using a quenching probe (MBQ-SP), with the i-densi system (Bio-RAY Inc.). ALK rearrangement was analyzed by immunohistochemistry (ALK detection kit; Nichirei Biosciences Inc.). Phosphorylation of MAPK (ERK1/2) was assessed by immunohistochemical analysis with the anti-phospho-p44/42 MAPK (ERK1/2, Th202/204) antibody (Cell Signaling Technology). Clinical and prognostic assessments were performed using the Kaplan–Meier method.

Results: Phosphorylated p44/42 MAPK was detected in 84 (76.4%) of the 110 patients. In the 79 patients whose phosphorylated p44/42 MAPK (m) had EGFR mutations, 8 (10.1%) had a KRAS mutation, and 2 (2.5%) had a BRAF mutation. The p44/42 expression level, which was assessed on the basis of the immunohistochemical score, was found to be lower in the patients with EGFR mutations and higher in the patients with a KRAS/BRAF mutation. The patients’ prognosis tended to worsen with increase in the phosphory-p44/42 expression level. These results showed that the activity of the MAPK signaling pathway increased to a greater extent because of KRAS/BRAF mutations than because of EGFR mutations and may have caused poor prognosis. No correlation was found between ALK rearrangement and the phospho-p44/42 expression level.

Conclusions: The expression level of phosphorylated MAPK was higher in the NSCLC patients with a KRAS/BRAF mutation than with EGFR mutations, and this higher activity level was associated with poor prognosis.

Investigating the Efficacy of the Irreversible Pan-ERBB Inhibitor PF-00299804 Using Paediatric Embryonal Tumor Models

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Background: Medulloblastoma (MB) and pineoblastoma (PB) are malignant paediatric brain tumours. Despite improved treatment regimens many children with these tumours remain incurable. Moreover, survivors are often left with devastating long-term side effects, highlighting the need for innovative therapies. Previous studies have revealed that over-expression of transmembrane receptors ERBB2 and ERBB4 is associated with poor clinical outcomes in children with MB. PF-00299804 is an irreversible pan-ERBB inhibitor, with anti-tumour activity in lung cancers that harbour deregulated ERBB receptors. We hypothesised that increased ERBB expression is associated with decreased sensitivity of MB cell lines to chemotherapy due to up-regulation of survival pathways and that PF-00299804 will improve the efficacy of conventional anti-cancer drugs.

Material and Methods: To evaluate the efficacy of PF-00299804, as a single agent and in combination with conventional chemotherapeutics, we have developed a panel of cell lines representing PB and various paediatric MB subtypes which were examined in vitro and in vivo using orthotopic xenograft. In addition, this study explored the utility of a novel model of MB, the MD2-SmoA1 mouse, which represents the Sonic Hedgehog subgroup of human MB. Immunoblot and immunohistochemical analysis for ERBB1–4 was used to determine ERBB pathway deregulation in our models. In vitro cell
CDK5 is involved in oxaliplatin response and resistance acquisition through regulation of STAT3 transcription factor.

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Introduction: Oxaliplatin (OXA) is a chemotherapeutic drug widely used in the treatment of colorectal cancer (CRC). Unfortunately, development of acquired resistance results in a major obstacle for effective treatment. In a previous work (Martínez-Carías et al. Mol Cancer Ther 2009;8(1):194–202), we found an up-regulation of cyclin-dependent kinase 5 (CDK5) mRNA in the HT29-derived OXA-resistant cell line, HTOXAR3. There are some evidences of an involvement of CDK5 in DNA-damage response through STAT3 and NF-κB signalling pathways, however, the role of this CDK in oxaliplatin response and resistance remains to be elucidated.

Materials and Methods: CDK5 silencing in HT29 and HTOXAR3 cells was performed by using siRNA technology with a pool of 3 Silencer Select validated siRNAs (s2826, s2825 and s2827, Ambion) and lipofectamine RNAimax (Invitrogen) diluted in Optimem medium (Invitrogen). Knockdown validation was done by Western Blotting (WB) and qPCR. To study the effect of CDK5 silencing in the resistance phenotype, an MTT assay was performed to compare the viability between CDK5-silenced cells (sCDK5) and those transfected with an unspecific siRNA (sNTC). Phosphorylation status of CDK5, STAT3 (Y705 and S727) and p65 was studied by WB at different times after treatment with different doses of OXA.

Results and Discussion: A CDK5 silencing over 85% was achieved at both mRNA and protein levels which lasted for more than 96 h and resulted in a 56% OXA IC50 reduction in HTOXAR3-siCDK5 cells as compared with sNTC or Mock (21.93 ± 19.30 μM). CDK5 was phosphorylated in its Y15 residue at 4 h post treatment with OXA, whereas this effect more evident in HTOXAR3 than in HT29 cells and independent of an increase in the levels of the CDK5 activator, p35. Activation of NF-κB in response to oxaliplatin was also increased in resistant cells. Following OXA treatment, STAT3 was phosphorylated on its C-terminal S727 but not in its Y705 residue, whereas this site was phosphorylated upon EGF stimulation. As a consequence of CDK5 silencing, levels of p-STAT3-S727 decreased suggesting that STAT3 is activated, at least in part, as a consequence of CDK5 interaction. After treatment with 30 μM OXA, phosphorylation on S727 was almost absent in HT29 cells as compared with HTOXAR3 cells, involving STAT3 pathway in the adaptive response of resistant cells to OXA treatment.

Conclusion: Cdk5-STAT3 play an important role in acquired resistance to OXA, probably by inducing the expression of genes involved in survival and/or DNA-damage repair. Further experiments are ongoing in order to confirm this hypothesis as well as the role of NF-κB in that respect. If confirmed, these results would lead us to speculate that Cdk’s and STAT3 inhibitors could be tested in the treatment of OXA-resistant CRC tumours.

Overcoming Trastuzumab Resistance in Breast Cancer by Targeting NF-κB Necrosis Factor Alpha

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Introduction: ErbB2 overexpression occurs in ~20% of invasive breast cancer and is associated with poor prognosis. Trastuzumab (T), a monoclonal antibody (Ab) against ErbB2, is given to patients with ErbB2-overexpressing breast cancer. However, the overall response rate is about 26% as a single agent and 40–60% when used in combination with chemotherapy. Failure to respond can be due to intrinsic or acquired resistance to T. We have previously demonstrated that tumor necrosis factor α (TNF) induces ErbB2 transactivation and is able to overcome the inhibitory effect of T on BT-474 and SKBR-3 cells proliferation through the activation of NF-κB. In this work we explored the effect of TNF blockage on the growth of breast cancer cells that exhibit intrinsic resistance to T, using the JIMT-1 cell line.

Material and Methods: TNF effect was blocked either by siRNA or by the TNF receptor2-FcIgG fusion protein, etanercept (E). For in vivo experiments, nude mice were injected with 3x10^6 JIMT-1 cells and treated with 5μg/ml E twice a week, 5mg/kg T once a week or both. Control group was injected with human IgG. Histopathological analyses were performed by H&E staining and by IHC for ErbB2 using A0485 Ab. For in vitro experiments, JIMT-1 cells were either transfected with siRNA or treated with 10μg/ml etanercept. T binding was detected by immunofluorescence and flow cytometry analysis.

Results and Discussion: Western blot (WB) showed that JIMT-1 cells produced the precursor and mature forms of TNF. JIMT-1 xenograft tumors treated with E or T showed no differences in growth compared to IgG-injected mice. However, simultaneous administration of E + T reduced tumor size by 54.1 ± 6.4% (P < 0.05). Histological examination revealed less mitotic figures and extensive hyalinization areas in the tumors from mice treated with both Ab in comparison to E, T or IgG groups. WB from tumor extracts of E + T group revealed a complete blockage of NF-κB activation as shown by inhibition of IκBα and p65 NF-κB phosphorylation. Interestingly, tumors from E-treated group showed strong ErbB2 staining when compared to T or IgG groups. In vitro blockage of TNF in JIMT-1 cells induced an augment of T binding. These results suggest that TNF blockage induces an enrichment of ErbB2 in plasma membrane that can be effectively targeted by T in JIMT-1 resistant cells.

Conclusion: Our results suggest that TNF blockage would be a promising tool to overcome intrinsic T resistance in ErbB2 overexpressing tumors.

Sample Quality Control Within Various Next Generation Sequencing Workflows Using a Unique, Automated Electrophoresis Platform

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This study evaluates the performance of the 2200 TapeStation System in various Next Generation Sequencing (NGS) workflows. Nucleic acid samples were checked for quality at different stages of various NGS protocols including pre- and post-shearing, post adaptor ligation as well as pre-hybridisation and post-hybridisation within the SureSelect target enrichment workflow. The data shows that this new automated electrophoresis system provides qualitative information that enables informed decision making in all downstream steps. By providing a range of ScreenTape consumables with standard and high sensitivities along with a tailored analysis package, the system is able to QC gDNA, fragmented DNA, whole genome libraries and target enriched libraries, presenting descriptive analyses at each stage for multiple sequence protocols. The data described here demonstrates that the 2200 TapeStation System has the range, sensitivity, precision and accuracy to meticulously QC samples within the NGS workflow.

Quality Control of DNA from Formalin Fixed Paraffin Embedded and Fresh Frozen Tissues Prior to Target-enrichment and Next Generation Sequencing

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There are over 400 million formalin-fixed paraffin-embedded (FFPE) tissue samples archived in biobanks worldwide. These diseased and normal tissue collections are valuable resources for molecular genetic studies. However, the challenges of DNA extraction from FFPE tissues, including formaldehyde cross-linking, degradation, and mixtures of single-stranded and double-stranded DNA, result in low amounts of usable high quality material for downstream assays. Hence, assessing the quality of samples to be processed for highly sensitive and costly applications, such as next generation sequencing, becomes a critical concern. On-chip, automated electrophoretic devices were evaluated for the characterization of FFPE and fresh-frozen DNA samples prior to and during target-enrichment and next generation sequencing workflows.
Bisnaphthalimidopropyl Derivatives as Antitumor Agents - Targeting SIRT2

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Background: Some polyamine derivatives, namely the bisnaphthalimidopropyl polyamines (BNIPs) may have potential as anticancer drugs. Indeed previous work from some of us had shown that the ability of these molecules to bind to DNA may contribute to their cytotoxicity. However, their precise mode of action has not been fully understood.

In a previous work we report for the first time the effect of the previously synthesised compounds, such as BNIPDaCHM (GB3) and NPA together with a new BNIP derivative [BNIP-3,4-DaDPM (CR1)], regarding their effect in the in vitro growth of a non-small lung cancer cell line (NCI-H460). In addition, the effect of the most potent compound (BNIPDaCHM (GB3)) was further investigated in an in vitro assay to verify if it inhibits sirtuins. This was further confirmed in silico.

Material and Methods: The effect of the previously described compounds, as well as that of a newly synthesised one [BNIP-3,4-DaDPM (CR1)] in the in vitro growth of a non-small lung cancer cell line (NCI-H460), was analysed with the sulphoradamine B assay. The effect of the most active compound on cell cycle profile and apoptosis was also studied, by flow cytometry following propidium iodide (PI) or Annexin V/PI staining, respectively. In addition, the expression of proteins involved in apoptosis (such as PARP, cleaved PARP and caspase-3) was also investigated, by Western Blot. An in vitro assay was also carried out in order to verify a possible interaction with sirtuins. In addition, the effect in the acetylation of tubulin was also analysed, by Western Blot. Finally, in silico studies were also carried out in order to verify the possibility of interaction with sirtuins.

Results: From the three compounds studied, BNIPDaCHM (GB3) was the most potent and caused a concentration-dependent alteration in the normal cell cycle profile of NCI-H460 cells. The induction of apoptosis by BNIPDaCHM (GB3) was confirmed by Annexin V/PI staining and by the increase in PARP cleavage and decrease in pro-caspase-3. Interestingly, this compound was found to inhibit SIRT2 in vitro and docking studies indicated that it is composed of an entire new SIRT2-inhibiting structural scaffold.

Conclusions: BNIP derivatives with a novel structural backbone, such as the one described for BNIPDaCHM (GB3), may have potential as anticancer agents that bind selectively to hSIRT-2.

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1006 A Compound From a European Medicinal Plant Induces Different Anti-Cancer Effects in Various Cancer Cells

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Background: Upon the discovery of an extract from a European plant with interesting anti-cancer properties, a bioactivity-guided isolation of active compounds was performed and molecular and cellular investigations to identify the implicated mechanism of action of the anti-cancer compound(s) were undertaken. The compound displays a high cytotoxic effect (nanomolar range) on several cancer cell lines whereas the effect on normal control cells was significantly weaker.

Material and Methods: The compounds were isolated by different chromatographic techniques and cytotoxicity was tested by MTT assays. In order to investigate the physiological state of the cells, cell viability tests as well as apoptosis detection by flow cytometry were performed. Furthermore, the cells’ morphology, motility and multiplication could be followed during the treatment with the compound using an imaging system. A set of microarrays was performed to analyze the gene expression in the different types of cancer cells.

Results: The compound induced cell death in leukemia cells (Jurkat) whereas it induced cytostasis in non-leukemia cancer cells. Moreover, the leukemia cells underwent apoptosis. Microarray analysis of the gene expression after a short treatment with the compound showed a set of differentially expressed genes in the two types of cancer cells (apoptosis versus cytostasis). Several of these genes are selected for in depth studies intended to reveal the initial mechanism of action of the compound as well as the key molecules for the apoptosis/cytostasis switch.

Conclusion: A compound from a European plant inducing differential anti-cancer effects in leukemia and non-leukemia cell lines has been identified. Gene expression analysis should allow to understand the mechanism of action of the compound leading to the apoptosis/cytostasis switch.

1007 Prodigiosin Increases Ubiquitin-ligases Gene Expression in Human Melanoma Cell Line SK-MEL-5

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Background: Prodigiosin (2-methyl-3-pentyl-6-methoxyprodigiosene, PG) is a backbonelike tripeptide structure. PG treatment has been reported to induce apoptosis in several cancer cell types with low cytotoxicity in non-malignant cells. Melanoma is an aggressive cancer with high metastatic potential and resistance to cytotoxic agents. Our group has recently described that PG induce apoptosis in SK-MEL-5 melanoma cells with a high inhibition concentration (IC50) value of 1 μM and inhibits AKT/TOR signaling pathway (Espona-Fiedler et al., 2012). Phosphorylated active form of AKT downregulates E3-ubiquitin-ligases by the inactivation of FOXO transcription factors (Sandri et al., 2004). Induction of two E3-ubiquitin-ligases mRNA expression leads to increased proteolysis via the ubiquitin-proteasome system (Gomes et al., 2001). The aim of this work is to identify the proteolytic systems involved in the cytotoxicity in human melanoma cells.

Material and Methods: For this purpose, SK-MEL-5 cells (vertical growth phase) derived from a metastatic site (axillary node) of a melanoma-bearing patient were used. Cells were treated with PG (IC50=1 μM) with or without DMSO (vehicle control) during 3 h. Reverse transcription and real-time PCR was used to analyze gene expression. Analysis of mRNA levels for the genes from the different proteolytic systems was performed with primers designed to measure the following gene products: ubiquitin, E2, C8 proteasome subunit; C2 proteasome subunit; E3-ubiquitin-ligases (TRIM63 and F-box protein 32), cathepsin B, m-calpain and 185 (invariant control). Moreover, we examined, by western blot analyses, total FoxO3a and phosphorylated FoxO3a (Ser253) protein levels in PG-treated cells.

Results and Discussion: E3-ubiquitin-ligases and E2 mRNA levels were increased in PG-treated melanoma cells compared to controls, whereas no differences were observed in both lysosomal (cathepsin B) and calcium-dependent (m-calpain) mRNA content. Furthermore, PG-treated melanoma cells showed a decrease of phosphorylated FoxO3a and an increase in total FoxO3a protein levels compared to control cells suggesting a higher transcription activity of downstream genes such as E3 ligases (TRIM63 and F-box protein 32).

Conclusion: These results suggest the involvement of the ubiquitin-proteasome proteolytic system in the cytotoxicity induced by PG in human melanoma cells. The inhibition of PI3K/AKT pathway by PG could activate FoxO transcription factor leading to an increase of E3-ubiquitin-ligases, and consequently triggering protein degradation involved in cell death mechanisms.

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1008 Characterization of Newly Established P-glycoprotein Over-expressing Multi-drug Resistant Human Cancer Cell Lines

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Background: Multidrug resistance (MDR) is a major obstacle to successful cancer treatment. Cancer cells manifesting MDR phenotype are cross-resistant to many structurally and functionally unrelated drugs. Over-expression of membrane transporter P-gp is the most common alteration in cancer cells with MDR phenotype. To resolve and overcome this problem in clinical practice, it is necessary to investigate the mechanisms of MDR and use a set of multidrug-resistant cell lines. Our study aimed to identify and characterize MDR cancer cell lines.

Material and Methods: We used three different MDR human cancer cell lines developed in our laboratory (NCI-H460R, DLD1-TxR, U87-TxR) and their sensitive counterparts (NCI-H460, DLD1, U87). Inverted-DAPI Banding was used for the construction of representative karyotypes. The mRNA expression of mdr1, which codes for P-gp was analyzed by RT-PCR. Flow-cytometric analyses were used to determine the level of P-gp expression. Western blotting was used to analyze gene expression. Analysis of mRNA levels for the genes of P-gp inhibitors (verapamil and tariquidar) on the accumulation of P-gp substrate (rhodamine 123). Detection of mdr1 single nucleotide polymorphisms (SNPs) was determined by real-time PCR.
Results: Cytogenetic data demonstrated that NCI-H460/R, DLD1-TxR and U87-TxR retained several karyotypic characteristics of their sensitive counterparts and also acquired novel structural or numerical chromosomal aberrations that may be related to resistance to chemotherapy. Gene expression analysis and flow cytometric analysis revealed that the most pronounced mechanism of MDR in resistant cell lines was the over-expression of P-gp associated with its activity. The homozygous mutations at position 2877G>T in mdr1 gene are present in all tested cancer cell lines. Only U87 and U87-TxR cell lines have the heterozygous mutations at the position 3435C>T. These mutations are involved in P-gp control regions. The P-gp inhibitors in MDR cancer cell lines. Indeed, our results showed that non-competitive P-gp inhibitor tariquidar was less effective in the inhibition of the P-gp efflux activity in U87-TxR cells.

Conclusions: Newly established resistant cancer cell lines represent models for studying the reasons for chemotherapy failure. Moreover, they are suitable for testing new therapies with the potential for overcoming MDR.

Targeting Chronic Myeloid Leukemia Stem/progenitor Cells by Effective Inhibition of a Novel AH1-1/BCR-ABL-JAK2 Interaction Complex

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Background: The tyrosine kinase inhibitor (TKI) imatinib mesylate (IM) induces clinical remission in most chronic phase CML patients. However, early relapse, acquired drug resistance, and inefficacy in eliminating leukemic stem cells are major problems associated with TKIs treatment for many patients. We have recently identified a novel protein interaction complex that forms in CML stem/progenitor cells between the oncogenes encoded by AH1-1 (Abelson helper integration site 1), BCR-ABL and JAK2. This protein complex contributes to the transforming activity of BCR-ABL both in vitro and in vivo and plays a key role in the IM response/resistance of primary CML stem/progenitor cells.

Material and Methods: Biological and molecular functions of AH1-1/BCR-ABL-JAK2 interaction complex, including response/resistance of CML stem/progenitor cells to TKI and a JAK2 inhibitor, were extensively investigated in primitive hematopoietic cells and leukemic stem/progenitor cells from CML patients both in vitro and in vivo.

Results: We have demonstrated that the WD40-repeat domain of AH1-1 is responsible for a physical interaction between BCR-ABL and AH1-1, while the N-terminal region of AH1-1 directly interacts with JAK2 in CML cells. Interestingly, loss of the interaction between AH1-1 and BCR-ABL and/or JAK2 significantly increased CML cell sensitivity to IM, as determined by cell viability, apoptosis and colony-forming cell (CFC) assays. Western and co-IP experiments demonstrated a significant reduction in p-BCR-ABL, p-JAK2 and p-STAT5 in AH1-1-transduced cells treated with IM plus TG101299 (TG), a JAK2 inhibitor, compared to cells treated with IM or TG alone. Treatment of CD34+ CML stem/progenitor cells with TKIs plus TG caused a significantly greater reduction in their viability, increased apoptosis, and reduced CFC production compared to TKIs or TG alone. Long-term culture initiating cell (LTIC-IC) assays showed that more primitive CML cells were also eliminated to a greater extent by combination treatment. Moreover, the combined treatment also effectively depleted more primitive primary CML cells capable of long-term, multilineage engraftment in immunodeficient NGS mice. Importantly, the combination treatment effectively targeted treatment-naïve CML stem cells from IM-resistant cells, resistant to eradication by TKIs alone.

Conclusions: These results point to the possibility of achieving improved therapeutic outcomes in CML patients before they develop TKI resistance. The inhibition of the fibronectin receptor with K34c decreases U87MG cell speed in single cell migration assays. Moreover, K34c also inhibits wound closure after scratch. K34c inhibitory effects were obtained on α5 expressing cells only, confirming its selectivity.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase, known to be a key mediator of integrin signalling and tumour cell migration. We show that K34c dose-dependently inhibits FAK phosphorylation on tyrosine 397, a major site for FAK activation. This was associated to the capacity of K34c to inhibit U87MG cell spreading and formation of focal adhesion on fibronectin.

Conclusion: Our results show that a small non-peptidic molecule, K34c, inhibits migration in correlation with α5 integrin expression. This process is tightly coupled to the dephosphorylation of FAK. These highlight the necessity to design new regulator of integrin α5/1 functions in the aim to broaden the range of targeted therapies in glioblastoma.

Synergistic Effects of PI3K or P38 MAPK Inhibition in Combination With Vandetanib Treatment in Glioblastoma Cells

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Background: A common obstacle in the treatment of glioblastoma is chemoresistance. A possible strategy to evade this problem is to combine chemotherapeutic drugs with agents inhibiting resistance mechanisms. The aim of this study was to identify molecular pathways influencing the sensitivity to chemotherapeutic drugs in glioblastoma cells, and evaluate the possibility of increasing the sensitivity by interfering with these pathways pharmacologically with selective inhibitors.

Methods: Global gene expressions were analyzed with microarray analysis and sensitivities to a panel of different chemotherapeutic drugs with the fluorometric microculture cytotoxicity assay (FMCA) in a set of six human glioblastoma cell lines. Gene expressions correlated to drug sensitivity were identified for each chemotherapeutic drug. Pathways that were enriched for synergism with the median effect method by Chou and Talalay. The aim of this study was to identify molecular pathways influencing the sensitivity to chemotherapeutic drugs with agents inhibiting resistance mechanisms. The chemoresistance. A possible strategy to evade this problem is to combine chemotherapeutic drugs with agents inhibiting resistance mechanisms.

Conclusion: Inhibition of PI3K or p38 MAPK may increase glioblastoma sensitivity to vandetanib.

The First Clinical Trial of Boron Neutron Capture Therapy Using 10B-para-boronophenylalanine for Treating Extramammary Paget's Disease

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Background: We previously showed that the fibronectin receptor expressing different levelsof a and b subunits. They play a major role in tumour cell migration and invasion. We previously showed that the fibronectin receptor expressing different levelsof a and b subunits. They play a major role in tumour cell migration and invasion. We previously showed that the fibronectin receptor expressing different levelsof a and b subunits. They play a major role in tumour cell migration and invasion.
can invade the dermis and may metastasize via the lymphatic system. The tumor has a predilection for apocrine gland-bearing areas, mostly the vulva, perianal, axillary, and penile. Wide surgical excision is the standard treatment of choice for EMPD; however, this treatment option is very invasive and may sometimes be unsuitable for elderly patients. Therefore, a minimally invasive therapy for treating EMPD needs to be developed. BNCT, an experimental form of radiotherapy, is based on the nuclear reaction between boron-10 ($^{10}$B) and neutron beam that produces alpha particles and lithium nuclei. The effect of these particles is primarily limited to boron-containing cells, because these particles have path lengths of approximately 1 cell diameter in tissues. Therefore, when $^{10}$B is selectively accumulated in tumor cells, tumor cells can be destroyed without any serious damage to the surrounding normal tissues.

Patients and Methods: We treated 2 elderly patients with EMPD, a 75-year-old man and a 73-year-old man, with BNCT. Both the patients had erythematous plaques and erosions on the genital regions. Histopathological examination showed tumor cells with abundant pale cytoplasms and large pleomorphic nuclei. These cells were arranged singly, in small groups, or formed irregular-shaped nests. These findings confirmed the diagnosis of EMPD. No signs of regional lymph node involvement and distant metastases were observed in both the cases. We chose BNCT for initial treatment against EMPD because both the patients strongly rejected surgery. The tumors were irradiated at the Kyoto University Research Reactor with thermal neutrons of 5 MW for 65 minutes and 78 minutes, respectively.

Results: Complete regression of the tumor was achieved in both the cases. Neither recurrence nor metastasis has been observed for more than 1 year after BNCT.

Conclusion: BNCT is a minimally invasive procedure and holds promise for EMPD treatment, especially for elderly patients who might be unable to perform daily activities after a surgery.

[1015] Efficacy of a Novel Peptide-drug Candidate Against Gamma-Synuclein Using a Mouse Model of Human Endometriosis

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Introduction: Endometriosis is estimated to affect 5–10% of women of reproductive age and causes infertility in about 50% of afflicted women. Gamma-Synuclein (SNCG) is a protein which is found to be upregulated in endometriotic lesions and is known to increase the transcription of Estrogen Receptor-α (ERα), Matrix Metalloproteinases and cell growth. In cancer, SNCG is correlated with significant cell proliferation and metastasis and with the development of resistance to anti-microtubule drugs. Here, we hypothesize that elevated levels of SNCG plays a role in the growth and establishment of endometriotic lesions. Therefore, blocking SNCG activity with a peptide inhibitor (TAT-P12) will prevent angiogenesis and development of human endometriotic lesions.

Methods: Molecular modelling, peptide depletion and biophysical studies were used in the discovery of peptide. Western blots were used to determine the amount of SNCG present in eutopic and eutopic endometrium. Immunohistochemistry (IHC) was performed to assess levels of SNCG in a TMA of endometriotic patients. Proliferation assays were performed on Human Umbilical Vein Endothelial Cells (HUVECs) that were treated with different concentration of TAT-P12 ranging from 600 nM to 2.5 nM/mL. In order to observe the effect of TAT-P12 on angiogenesis, in vitro angiogenesis assays were conducted using HUVECs that were treated with different concentrations of TAT-P12. Female BALB/c-Rag2−/−Il2rg−/− aliphoid mice (n = 12) were primed with subcutaneous human estrogen pellets five days prior to intraperitoneal injection of human endometrial fragments. After 7 days, mice were treated with TAT-P12 (10 mg/kg, n = 6) or vehicle (n = 6) for 21 consecutive days. At the end of treatment, all the experimental mice were sacrificed to assess vascularization of the endometriotic lesions using CD31 IHC. Side effects of TAT-P12 on several organs including liver, spleen, kidney, uterus and ovary were evaluated by H&E staining.

Results: A twelve amino acid novel peptide (P12) has been discovered which binds to SNCG with high avidity. Unique cell penetrating vectors of P12 have been designed. We found high expression of SNCG in ectopic endometrium compared to eutopic endometrium. Treatment with TAT-P12 inhibited proliferation of HUVECs in between 24–48 hours. TAT-P12 was unable to dose dependently block angiogenesis in HUVECs. Experimental mice treated with TAT-P12 showed reduced vascularisation of endometriotic lesions. No abnormal side effects were observed in other organs such as liver, spleen, kidney, uterus and ovary. Studies are currently in progress to determine extent of apoptosis endometriotic lesions in TAT-P12 treated versus control mice.

Conclusion: The data suggests that SNCG may play a role in the pathogenesis of endometriosis and blocking SNCG using P12 may inhibit lesion growth in women by targeting angiogenesis. This peptide has a strong potential to be a new therapeutic against metastatic cancers.

[1016] NPM-ALK I1171T Mutation Confers Resistance to Crizotinib in Anaplastic Large Cell Lymphoma

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Introduction: One of the most important breakthroughs in oncology in the last few years has been the discovery of anaplastic lymphoma kinase (ALK) gene rearrangements in various types of cancers. Identification of ALK mutations as oncogenic drivers in certain malignancies led to the development of small molecule ALK inhibitors with one of them – crizotinib, already approved for the treatment of ALK-positive NSCLC patients. Targeted therapies with kinase inhibitors in precisely selected groups of patients may bring encouraging results but unfortunately are often accompanied with acquiring resistance to the treatment and patients’ relapse. The key issue then is to predict the possible mechanism of resistance in order to provide patient with an alternative therapy. One of the most frequent mechanisms of resistance to crizotinib therapy in NSCLC patients are resistance mutations correlated with resistance to crizotinib in NSCLC. We decided to investigate potential mechanism of resistance in NPM-ALK positive anaplastic large cell lymphoma (ALCL). As a model we used Karpas299 cell line which carries NPM-ALK fusion and is highly dependent on ALK kinase pathway. The cells were treated with increasing concentrations of crizotinib and when exceeded an order of magnitude the IC$_{50}$ value ALK gene was sequenced in order to indentify potential mutations. In addition, the same procedure was applied using CH5424802 ALK inhibitor.

Results and Discussion: In the following study we established ALCL cell line resistant to crizotinib. We confirmed the mechanism of resistance to be the ALK mutation I1171T. Additionally we showed that the I1171T positive cell lines were also resistant to another ALK inhibitor CH5424802. The results suggest that resistance related to the mutation in 1171T position of ALK might not be overcome by the treatment with other ATP competitive ALK inhibitors in second line treatment.

We also managed to establish ALCL line resistant to CH5424802. We did not identify any mutation in the line so far and it is highly plausible that the resistance in this case results from activation of alternative signaling pathway.

Conclusion: Our results reveal that I1171T mutation can comprise one of the mechanisms of acquired resistance to crizotinib in ALCL patients. Monitoring of ALK status during therapy with ALK inhibitors is a key issue in light of treatment success.

[1017] Human Dedifferentiated Liposarcomas Growth Inhibition by SAR299155, a Potent and Selective Disruptor of the MDM2-p53 Interaction

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The tumor suppressor protein p53 plays a fundamental role in protecting cells from malignant transformation through the induction of cell cycle arrest and apoptosis in response to diverse stresses. MDM2 guards the switch of p53 levels and activities by binding to the transactivation domain of p53 and by acting as a p53-specific E3 ubiquitin ligase. The amplification of MDM2 gene has been observed in several types of cancer, including 100% of human dedifferentiated liposarcoma. The disruption of the interaction between p53 and MDM2 with small molecules has demonstrated encouraging single agent anti-tumor activities in vitro and vivo. Here, we have explored the effect of SAR299155, a selective MDM2-p53 antagonist [1] on p53 pathway and the resulting phenotypic changes observed in human dedifferentiated liposarcoma cell lines (DDLP5).

MDM2 gene copy number and TP53 status were determined in 3 human DDLP5 cell lines with treated various SAR299155 concentrations (0.01−10 μM) and 24h incubation. The most p53-dependent biomarkers such as p53 itself, p21, MDM2, PUMA and MIC-1 was studied. All DDLP5 cell lines exhibited wild type TP53 status and genomic amplification of MDM2. Cell viability was decreased in a dose dependent manner with IC$_{50}$ values of 0.067 to 0.404 μM, depending on the cell line. While SAR299155 induced apoptosis in DDLP5 cell lines, it also significantly modulated p53 pathway, as monitored by p53 stabilization and induction of p53 transcriptional target genes at protein and transcript levels.

These data demonstrate that SAR299155 is a highly potent and specific inhibitor of MDM2 with activity in genetically characterized dedifferentiated liposarcoma.
liposarcoma cells. Further study and clinical development in this disease is warranted.

Reference(s)

1016 Disulfiram, an Antialcoholism Drug, Targets Breast Cancer and Glioblastoma Stem-like Cells
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Introduction: Cancer derives from cancer stem cells (CSCs). Conventional anticancer drugs target the differentiated tumour bulk but fail to eradicate CSCs which become the source of tumour recurrence. Therefore development of anti-CSC drugs will improve the outcomes of cancer therapeutics.

The low lost and high cost (15 years and US$1 billion/one new drug) is the dilemma of current drug development. There are more than 25,000 FDA approved drugs. Considering the known pharmacokinetics and safety profiles, any newly identified application of an existing drug would facilitate rapid evaluation in phase II clinical trials. Therefore repurposing of old drugs has become a very attractive strategy for drug development. We have previously demonstrated that Disulfiram (DS), a clinically used antialcoholism drug, enhances anticancer drug-induced apoptosis. DS is an inhibitor of aldehyde dehydrogenases (ALDHs), the functional markers of CSCs. Knocking down ALDH genes inhibits the growth of ovarian, breast, lung cancer and leukemia cell lines and sensitizes CSCs to conventional anticancer drugs.

This study is aiming to investigate the anti-CSCs activity of DS.

Materials and Methods: MCF7, MDA-MB-231, T47D BC cell lines and U87MG, U251MG, U373MG GBM cell lines were examined. MTT cytotoxicity assay, Western blot, EMISA, laser assay, manono- and neuro-sphere formation and CSCs marker analysis were used in this study.

Results: DS was cytotoxic to BC and GBM in copper-dependent manner. In copper concentration of 200 μM (Cu), DS to cancer cell lines were 200–500 nM. The chemosensitizing effect of DS on 3 anticancer agents, gemcitabine (dFdC), doxorubicin (DOX) and paclitaxel (PAC), was determined in BC and GBM (dFdC only) cell lines. In combination with DS/Cu, the cytotoxicity of dFdC, DOX and PAC was significantly enhanced (DOX: 8–11-fold; dFdC: 1.2–23.5-fold; PAC: 4–10-fold). The synergistic effect between DS and the conventional anticancer drugs was confirmed by CI-isobologram. Clonogenic assay showed DS/Cu-inhibited clonogenicity of BC cells. The sphere forming ability and the ALDH1-1 and CD133+CD44+ (in BC) CSCs population in the spheres were significantly inhibited by exposure to DS/Cu. DS simultaneously activated and inhibited ROS-JNK and NFκB activity. Our data suggested that further studies may lead DS into cancer therapeutics.

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1017 Erlotinib Potentiates Cetuximab-dependent Cell Cytotoxicity in Egfr Wild Type Nsclc Cell Lines
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Introduction: The oral epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKi) Erlotinib (Tarceva, OSI Pharmaceuticals, Melville, NY) was proven to extend overall survival (OS) in patients after four cycles of platinum based double-agent chemotherapy and it is approved by the US Food and Drug Administration (FDA) for treatment after first- or second-line chemotherapy. Despite these modest gains in NSCLC treatment, better therapies are needed. Currently, two different papers have proposed the successful combination of TK inhibitors and mAbs, directed to the same target. We hypothesized that the combination Erlotinib and Cetuximab or Trastuzumab may be effective in selected NSCLC cell lines.

Material and Methods: a panel of NSCLC cell lines with EGFR wt were cultured as recommended. Cell viability was determined by MTT assay. EGFR and ErbB2 expression was evaluated by Western blot analysis. The protein membrane expression was quantified by flow cytometry. Human peripheral blood mononuclear cells (PBMC) were obtained from healthy donors using Histopaque (Sigma Aldrich). The effect of mAb was determined by incubating NK isolated cells (Milenyi Biotech) activated with IL-2 100 U/ml with cancer cells treated with vehicle or 0.5 μM Erlotinib for 24h.

Results and Discussion: After 48h of Erlotinib treatment only NSCLC cell lines with IC50 <1 μM (Calu-3, H322 and H292) showed an increase of EGFR and ErbB2 protein levels; the cancer cell lines with higher IC50 (H1299, AS45, Calu-1) didn’t show any change in these receptors. The flow cytometry confirmed an increase of protein at membrane levels indicating the rationale of the combination of Erlotinib with specific mAbs (Cetuximab and Trastuzumab).

To better determine the mAb role an ADCD assay was performed. After isolation from healthy donors, NK cells were seeded in the question whether Calu-3 cells treated with Erlotinib or vehicle were added after 16h with/without Cetuximab and the LDH release was quantified: the data obtained confirmed the hypothesis that the increased protein levels after TKI treatment potentiated the Cetuximab effect.

Conclusion: EGFR wt NSCLC cell lines responsive to Erlotinib showed an increase of EGFR and ErbB2 proteins after Erlotinib treatment. The sequential combination of Erlotinib and mAbs represents a potential strategy to improve maintenance therapy in NSCLC patients.

1021 A New Protocol for Determining Intracellular Concentrations of Antitumor Compounds – How to Calculate a Real and Effective IC50
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Background: IC50 calculation is crucial for estimating efficacy of antitumor compounds. However, behind the simple concept of IC50 there are subtle differences in the meaning of IC50 which become the source of tumour recurrence. Therefore development of anti-CSC drug will improve the outcomes of cancer therapeutics.

Methods: For cell imaging a Leica TCS AOPS SP2 confocal microscopy with 40x oil immersion objective and a 496 nm argon/krypton ion laser was employed. Cells were stained with acridine orange (AO) and signal was detected at 506–590 nm. An average of 20 2-series profiles were collected (1 μm interval). Finally, IMARIS 3D software was used for 3D reconstruction. Several normal and cancer cell lines were used including NIH3T3 fibroblasts; normal prostate PNT1A; ovarian CHO cells; prostate cancer cell lines LNCaP, PC-3 and DU-145 cells; breast cancer MCF-7 cells, cervical carcinoma HeLa, murine melanoma B16-F10, mouse macrophage RAW 264.7 and rat glioma C6 cells.

Results: We determined that AO staining was suitable for cell volume determination since the influence of slide width in the ‘z’ axis in final measurements of cell surface average between scans (1–30) was virtually none. Operator (reproductibility) was shown to have no influence in the final measurement of several cell lines. Error on cell volume measurements was less than 4.5%.

Conclusion: Cell volume estimated under this method is more realistic as cell manipulation is minimized. The protocol represents a simple, fast and accurate tool for measuring cell volume in several cell types using a common CLSM. Combination of this with analytical techniques provides a precise intracellular concentration of antitumor compounds. Method proposed here may fill the gap and offers an easy and inexpensive way to overcome cell volume determination difficulties.

1022 Sensitivity of Prostatic Neuroendocrine like Cells to Anti-tumor Drugs
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Background: Role of neuroendocrine (NE) differentiation in prostate cancer progression is still uncertain. NE cells might contribute to tumor growth by releasing mitogenic factors but also because they display lower resistance to chemo/radiotherapy. Some experimental paradigms including androgen withdrawal or antioxidant induction induce an increase in NE markers in androgen dependent prostate cancer cells, becoming the so called NE-like cells. However, relative sensitivity of NE-like cells to antitumor drugs has not been evaluated. Since specific phenotypic changes might be responsible for cell sensitivity to antitumor compounds, we studied cell survival of NE-like cells generated after androgen withdrawal or antioxidant incubation.
Material and Methods: NE-like clones from androgen-dependent LNCaP cells were generated by chronically growth with endogenous antioxidant melatonin (LNCaP FBSch) or melatonin combined with 5α-dihydrotestosterone (LNCaP MELp12). We tested in vitro the anti-proliferative activity of the tested compounds was determined by HPLC. The structures of final compounds were confirmed by NMR spectroscopy and HRRMs spectroscopy.

Conclusions: The anti-proliferative activity of the tested compounds was higher than Dp44mT. Moreover the ability to induce cellular iron release and preventing iron uptake from Tf in the same level than Dp44mT. This demonstrated high chelation efficiency in terms of mobilizing cellular iron upon phosphorylation. Reducing HuR levels diminished the apoptotic response to doxorubicin. We identified HA1004, AG494, U0126, AG490, Rottlerin and Erlotinib as iron chelators in vitro that inhibit HuR cytoplasmic accumulation and pointed to PKCδ, Rho kinase and ERK as potential HuR regulators. Among the hits rottlerin showed to be the most effective in blocking HuR nuclear export and in having correspondingly antagonistic effects with doxorubicin on cell toxicity. Co-immunoprecipitation of PKCδ and HuR upon doxorubicin confirmed the validity of HCS indications. In vitro selected doxorubicin resistant MCF-7 cells (MCF-7/doxR) expressing the multidrug resistance (MDR) related ABCG2 transporter, we observed a significant HuR downregulation that was paralleled by a corresponding downregulation of HuR targets as TOP2A and by loss of rottlerin toxicity. Restoration of HuR expression in these cells sensiitized MCF-7/doxR cells to doxorubicin, reactivating the apoptotic response.

Conclusions: The present study shows that HuR is necessary to elicit the apoptotic cell response to doxorubicin, that restoration of HuR expression in resistant cells sensitizes them to the action of this drug. Moreover we suggest a novel mechanism of pharmacoresistance based on the interplay among the doxorubicin target TOP2A, its post-transcriptionally regulator HuR and the signaling control of PKCδ.

[1025] Targeting Malignant Peritoneal Mesothelioma – Therapeutic Potential of Synthetic Retinoids

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Malignant peritoneal mesothelioma (MPM) is a rare but aggressive peritoneal surface malignancy accounting for ~30% of all mesotheliomas and known for unfavorable prognosis and poor response to conventional treatment. STO and MESSOI are two cell lines established in our laboratory from fresh surgery specimens and maintained in vitro in presence of serum. Previous unpublished data from our laboratory demonstrated that the presence of the putative stem marker ALDH1 was retained in STO cells (~75%) while it was 4-fold higher in MESSOI growing as non-adherent spheres compared to the original cell population (20% vs 5%). Since ALDH1 family of enzymes are involved in metabolism of retinoids (molecules structurally related to vitamin A), we explored the antitumor activities of natural (all-trans retinoic acid (RA), 13-cis-RA (cis-RA)) and synthetic retinoids (fenretinide (4-HPR) and its metabolite 4-oxo-fenretinide (4-oxo-4-HPR)) on STO and MESSOI cells.

Among the tested retinoids, 4-HPR and 4-oxo-4-HPR resulted very effective in inducing cell growth inhibition and apoptosis in STO and MESSOI cells (i.e. 50% at 72h; 50 and 0.6 μM for STO and 4 and 1.6 μM for MESSOI, respectively). The study of the molecular events underlying 4-HPR and 4-oxo-4-HPR activity in MPM cells showed that the retinoids caused reactive oxygen species (ROS) generation, however, their cytotoxic effects were independent from the induced oxidative stress. In fact, the abrogation of ROS generation by vitamin C did not protect from of the retinoids cytotoxic effects. With regard to the peculiar mode of action of 4-oxo-4-HPR (multipolar spindle formation and mitotic arrest), in both cell lines the retinoid was able to exert autocrine activity. The effects of 4-HPR and 4-oxo-4-HPR on stem cell properties were then evaluated. In MESSOI cells growing as non-adherent spheres in serum-free medium, both retinoids reduced sphere formation, indicating that they may target the putative subpopulation of tumor-initiating cells. In STO cells, 4-HPR and 4-oxo-4-HPR caused a marked decrease in the expression levels of the stem markers Sox2 and NANOG, suggesting that they can modulate in vitro stem cell features. Even though the exact mechanisms by which these retinoids exert their activities are not yet fully elucidated, these preliminary data indicate that 4-HPR and its active metabolite are able to efficiently target MPP and suggest a therapeutic potential for these retinoids for the treatment of the disease.

[1026] AF1q – a Novel Mediator of Basal and 4-HPR-Induced Apoptosis in Ovarian Cancer Cells

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Fenretinide (4-HPR) is a synthetic retinoid that exhibits potent antitumor and chemopreventive activities against different malignancies. We previously showed that in ovarian cancer cells, 4-HPR induces apoptosis through a signaling cascade starting from reactive oxygen species (ROS) generation, however, their cytotoxic effects were independent from the induced oxidative stress. In fact, the abrogation of ROS generation by vitamin C did not protect from of the retinoids cytotoxic effects. With regard to the peculiar mode of action of 4-oxo-4-HPR (multipolar spindle formation and mitotic arrest), in both cell lines the retinoid was able to exert autocrine activity. The effects of 4-HPR and 4-oxo-4-HPR on stem cell properties were then evaluated. In MESSOI cells growing as non-adherent spheres in serum-free medium, both retinoids reduced sphere formation, indicating that they may target the putative subpopulation of tumor-initiating cells. In STO cells, 4-HPR and 4-oxo-4-HPR caused a marked decrease in the expression levels of the stem markers Sox2 and NANOG, suggesting that they can modulate in vitro stem cell features. Even though the exact mechanisms by which these retinoids exert their activities are not yet fully elucidated, these preliminary data indicate that 4-HPR and its active metabolite are able to efficiently target MPP and suggest a therapeutic potential for these retinoids for the treatment of the disease.

Poster Sessions
plasmid expressing an AF1q siRNA and a scrambled nonsilencing siRNA, AF1q was found functionally involved in 4-HPR-induced apoptosis in A2780, an ovarian cancer cell line, thus suggesting the redinioid growth inhibitory and apoptotic effects. The inhibition in turn of the signaling intermediates of the 4-HPR apoptotic cascade, obtained by pharmacological inhibitors (the antioxidant vitamin C, the ER stress inhibitor salubrinal, and the JNK inhibitor SP600125) and by PLAB silencing, showed that AF1q upregulation was dependent on prior generation of ROS, induction of ER stress response, JNK activation, and PLAB upmodulation. Interestingly, we found that direct overexpression of AF1q, by transient transfection with a Green Fluorescent Protein-tagged AF1q vector, in the absence of external stimuli, increased apoptosis in ovarian cancer cell lines. This study expands the knowledge of 4-HPR mechanism of action which is not yet completely elucidated, identifying AF1q as a novel mediator of the retinoid anticancer activity. According to the apoptotic role suggested by our observations, AF1q was previously reported to be involved in the ROS-dependent doxorubicin- and γ-radiation-induced apoptosis. In addition, our results demonstrate for the first time, that AF1q plays a role in the onset of basal apoptosis in ovarian cancer cells, thus providing new information about the activity of this protein which biologic functions are mostly unknown.

**CD133 (Prominin-1) as a Therapeutic Target in Metastatic Melanoma**

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**Introduction:** The development of metastasis is by far the major cause of melanoma deaths. Metastatic melanoma is largely refractory to all currently available systemic therapies, with infrequent durable responses to systemic therapy. We have previously shown that down-regulation of CD133 severely reduced melanoma metastatic capacity (Rappa et al., Stem Cells 26: 3008–17, 2008). We hypothesize that CD133 contributes to melanoma cells pro-metastatic behavior and is therefore an excellent target to prevent melanoma dissemination.

**Materials and Methods:** We have employed in this study human FEMX1 melanoma cells, originally derived from a lymph node metastasis of a melanoma patient. This cell line, tumorigenic and metastatic, expresses on its cell surface relatively high levels of CD133 (400,000 molecules/cell on average). We employed antibody clones AC133 and AC141, recognizing different epitopes of human CD133. Golgi was stained by a baccilion expressing a signal peptide for N-acetyl-galactosaminyl transferase fused with GFP under the control of a CMV promoter.

**Results:** Upon binding of both AC133 and AC141 antibodies to CD133 on the surface of FEMX1 cells, the antibodies were progressively internalized and transported to the Golgi, with a complete accumulation in the Golgi between 12 and 24 h. We conjugated the AC133 antibody with the toxin saporin. For non-targeted saporin control, we used saporin-pre-immune mouse IgG antibodies. In the presence of anti-CD133 monoclonal antibody, saporin-conjugated secondary antibody was toxic to CD133-expressing FEMX1 cells, but not to control human fibroblasts. The saporin-conjugated AC133 antibody was much more effective on FEMX1 cells than on CD133 knock-down cells, expressing only 15,000 CD133 molecules/cell, while the pre-immune conjugate showed modest toxicity only at 2-log higher concentrations. These data indicate that, as expected, the level of expression of CD133 is an important determinant of the immunotoxic activity.

**Conclusion:** We expect that, as result of our study, anti-CD133 agents will be identified and preclinically validated for both efficacy and safety, the final aim being to test them in appropriate clinical trials for melanoma patients. Due to the expression of CD133 in many types of cancer, our findings will also have a potential impact on the treatment of different types of malignancies.

**A Novel Lipoplex Formulation for Targeted Cancer Gene Therapy**

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**Background:** Hepatocellular carcinoma (HCC) is the third largest cause of cancer mortality worldwide. Currently the primary HCC treatment options have many limitations, therefore there is an urgent need to develop new antimaluralstrategies, gene therapy being considered as a promising approach for treatment of this devastating disease. Regarding this, the main goal of this study was to develop a new gene delivery system with the capacity to specifically and efficiently deliver genetic material into HCC.

**Material and Methods:** The biological activity and specificity of the different lipoplex formulations were evaluated in a hepatocellular carcinoma cell line (HepG2 cells) using different techniques: luminescence, flow cytometry and fluorescence microscopy. Cell Binding and uptake of lipoplexes were measured by fluorescent dihydrothymidinelabeled complexes. Cell viability studies were conducted using the Alamar Blue assay.

**Results:** The results obtained in transfection studies with HepG2 cells, showed that the association of asialofetuin (ASF) to lipoplexes, prepared with 1-palmitoyl-2-oleoyl-sn-glycero-3-ethyphosphocholine (EPOPC) :cholesterol (Chol) cationic liposomes, promotes a substantial increase in their biological activity. This enhancing effect was ASF concentration-dependent and corresponded to a 40-fold increase in the best condition. This potentiation induced by ASF was observed not only by the increase of the transgene expression but also by the enhancement of the percentage of transfected cells. The biological activity obtained with these lipoplexes was much higher than that observed with highly efficient commercial formulations. In the presence of galactose, it was observed a substantial decrease in the biological activity of lipoplexes, most probably due to galactose competition with the ASF for the binding to the asialoglycoprotein receptor (ASGP-R). In addition, it was found that the association of ASF to lipoplexes promotes an increase in their cell binding and uptake that were inhibited in the presence of galactose.

**Conclusions:** Overall our results suggest that the potentiation of the biological activity induced by the ASF association to lipoplexes is due to its specific binding to the ASGP-R overexpressed in HCC. Regarding this, this novel lipoplex formulation, containing ASF, could be an interesting system that may be useful for the development of specific antitumor strategies against HCC.

**ATP-binding Cassette Transporter Inhibition by Tyrosine Kinase Inhibitor Nilotinib**

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**Background:** Nilotinib (Tasigna®), a tyrosine kinase inhibitor designed to inhibit BCR-Ab1 activity in chronic myeloid leukemia, inhibits many other kinases involved in tumor proliferation, apoptosis and differentiation, and is a potential blocker of Hoechst dye exclusion in side-population (SP) cells, which is mediated by ATP-binding-cassette drug-transporters (ABC-transporters). ABC-transporters are one of several mechanisms responsible for innate and acquired drug resistance in tumors. The transporters most frequently related to drug resistance include multidrug resistance protein 1 (MDR1, P-glycoprotein), multidrug resistance-associated protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2). Pancreatic cancer is notoriously known for poor response to chemotherapy and thus we investigate the effect of Nilotinib on pancreatic cancer cell proliferation, SP dye efflux and ABC-transporter expression in gemcitabine-sensitive and -resistant cells in vitro.

**Methods:** Cell lines Capan-1, Panc-1, Panc-1-GR (resistant to 1μM gemcitabine) and Capan-1 GR (resistant to 10μM gemcitabine), were treated with Nilotinib concentrations of 10 nM to 10μM. Proliferation was assessed by cell counting, cell cycle distribution by flow cytometry using propidium iodide and ABC-drug pump mediated efflux by Hoechst-dye-exclusion-assay. Gene expression was analyzed by qPCR and immunofluorescence. Results were confirmed in primary pancreatic cancer cell lines.

**Results:** In Capan-1 and Panc-1 cells, viability dropped to 33% and 15% following 72h of 10 μM Nilotinib compared to untreated controls, and to 45% and 33% for Capan-1-GR and Panc-1-GR cells. Cell cycle distribution showed G1 arrest in Panc-1 cells after 72h and increased sub-G1 events in all other cell lines. In Panc-1 cells SP was reduced to 0.07% in the presence of 1μM Nilotinib (vs. 2.2% in unblocked controls) and to 0.1% (vs. 11.5% in unblocked controls) in Panc-1 GR cells. Blocking efficiency was superior to 50μM Verapamil in all cases. ABCG2 expression was reduced to 0.5-fold in Capan-1 GR and Panc-1 cells after 72 hours and increased 2.2-fold in Capan-1 cells. ABC-transporter inhibition was confirmed in five primary pancreatic cancer cell lines.

**Conclusion:** Nilotinib inhibits proliferation in both gemcitabine sensitive and resistant cells. It effectively blocks ABC-transporter mediated drug efflux, most prominently in GR cells with an increased SP population, induces differential changes in ABCB1, ABC1 and ABCG2 expression on mRNA levels and inhibits BCRP/ABCG2 expression on the cell membrane. This new mechanism could be useful in combination therapies against ABC-transporter expressing tumors.

**Differential Gene Expression Profile in Response to Treatment With JAK2 Inhibitors – Ruxolitinib, SAR320253 and LY2785454**

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**Introduction:** JAK2 kinase inhibitors comprise a promising treatment strategy in several haematological malignancies including primary myelofibrosis (PMF), polycythaemia vera (PV) or essential thrombocythaemia (ET). Considering the number of patients suffering from myeloproliferative disorders carry a point mutation in exon 14 of the JAK2 gene (valine-to-phenylalanine at codon 617) which is assumed to be an oncogenic driver in these cases.
One of the leading compounds in this field – Ruxolitinib, discovered by Incyte, have been recently approved for the treatment of PMF and a few other hematological malignancies under investigation in clinical trials. In the present study we investigated the mechanism of action of Ruxolitinib and two other JAK2 inhibitors being developed by Sanofi and Eli Lilly – SAR302503 and LY2784544, respectively.

**Material and Method:** We compared the profile of C-MYC, VEGF, BCLXL, CCND1 and MMP2 genes expression in two different cell lines treated with the three mentioned JAK2 inhibitors. The genes were chosen according to their dependence on JAK/STAT pathway as well as to their known contribution to different stages of oncogenesis. As an in vitro model we used HEL-92.1.7 cell line that carries JAK2 [V617F] mutation and HL-60 with wild type JAK2. Both lines were also analyzed on FACS to evaluate the influence of each inhibitor on the cell cycle.

**Results and Discussion:** In our gene expression studies we found that only Ruxolitinib had no effect on HL-60 cells suggesting high selectivity of the compound. As expected, Ruxolitinib evoked significant decrease in CCND1, BCLXL and VEGF expression in HEL-92.1.7 cells confirming its anti-proliferative JAK/STAT inhibiting role. On the other hand we showed that SAR302503 and LY2784544 evoked changes in expression of CCND1, BCLXL and VEGF in HEL-92.1.7 cell line. These results suggest that SAR302503 and LY2784544 have additional off-target properties that probably are the effect of lower selectivity of these inhibitors. Moreover we analyzed the cell cycle of the treated HEL-92.1.7 cells and we found that SAR302503 evoked significant decrease in the number of cells in phase S and G2/M, whereas LY2784544 triggered accumulation of cells arrested in G2/M phase. Concurrently we did not notice any changes in the cell populations according to their cell cycle status after Ruxolitinib.

**Conclusion:** These results suggest that the three chosen JAK2 inhibitors have different mechanism of action which results in differences in their biological and clinical effects. All investigated inhibitors have been shown in clinical trials to entail different dose limiting toxicities what confirms our in vitro observations. Profound investigation of kinase inhibitors using several approaches can be helpful in predicting their potential off-target activity and possible side effects.

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Sonic Hedgehog Signaling Regulates Multi-drug Resistance and Affects Liposomal Doxorubicin Effect in Human Breast Cancer Cells

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**Background:** Sonic hedgehog (Shh) signaling pathway has been reported to associate with resistance to chemoradiation in esophageal adenocarcinoma and resistance to multiple structurally unrelated chemotherapeutic agents. Mutated JAK2 (JAK2M) is a main target of the JAK/STAT pathway, e.g. the ATG-binding cassette (ABC) proteins, reduces intracellular drug accumulation and causes the failure of treatment against cancers.

**Material and Methods:** To clarify the regulatory effect of Shh on drug resistance in breast cancer, JAK2F (HER2 ER2), MDA-MB-231 (HER2 ER2 PR2), and BT-474 (HER2 ER2) cells were tested. The doxorubicin (DOX)-resistant (MCF-7R) cells were developed with up-regulation of p-glycoprotein (P-gp). Treatment with siRNA directed against p-glycoprotein did not show significant effect on the MCF-7R cells. However, the downregulation of P-gp expression in the MCF-7R cells was also observed with the expression of Shh and Smoothened in both MCF-7S and MCF-7R cells. These results indicate that the role of Shh signaling in MDR might be upstream to P-gp function, partially reversed the DOX and VNR resistance in the MCF-7R cells. Moreover, the expression of N-Shh and nuclear translocation of Gli-1 was upregulated in MCF-7R cells as compared to the MCF-7S clone.

**Results:** The Shh-peptide inhibited the growth of human lung adenocarcinoma cells by 2.8-fold and 3.7-fold, respectively, correlated with ROS production and apoptosis (46% and 38% dead cells, respectively) observed after 24 hours of treatment. On the other hand, 6-hydroxyhexyl isothiocyanate at the same concentration caused similar reduced glutathione depletion but a cell cycle distribution analysis revealed an arrest in G2/M phase with no caspase-3 activity. Most isoiothercyanates were equally active on both cell lines, however some differences in mechanism of action were observed (e.g. zec-butyl isothiocyanate (concentration = 10mM) caused only a cell cycle arrest in G2/M on LoVo cell line but apoptosis on LoVoDX).

**Conclusions:** Current experiments over the biological activity of naturally occurring isoiothercyanates clearly indicates that chemical structure of these compound strongly influence not only their overall antiproliferative activity, but also their main molecular targets and mechanism of action. Studies revealed that some previously not tested compounds like 6-metoxysulfinly isothiocyanate or 6-metoxysulfinly isothiocyanate benzoate possess high antiproliferative activity. These observations can prove useful in future experiments on the isoiothercyanates potential in cancer therapy.

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A Novel Non-COX-inhibitory Sulindac Derivative Induces Autophagic Cell Death in Lung Adenocarcinoma Cells

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**Background:** In this study, we describe a novel non-COX inhibitory derivative of NSAID sulindac, referred to as sulindac sulfide amide (SSA), which was found to induce the growth of lung adenocarcinoma cell lines primarily through the induction of autophagy with or without apoptosis.

**Materials and Methods:** The H1299 and H232 cells were obtained from ATCC and cultured in RPMI medium containing 5% fetal bovine serum and 1% penicillin-streptomycin. Induction of cell growth was determined using the luminescent CellTiter-Glo Assay (Promega). Induction of apoptosis was determined using the luminescent CytoGlo (Promega) and the Annexin V/PI apoptosis kit (Invitrogen). For autophagic activity was measured by EdU (5-ethyl-2'-deoxyuridine) incorporation assay kit (Invitrogen). For cell cycle analysis, cells were stained with propidium iodide and DNA content determined by flow cytometry. For glutathione assay, L-Glutathione reagent and the Annexin V/PI apoptosis kit (Invitrogen). For neutralized medium introduced with carriers and staining with hematoxylin & eosine. For analysis of the TAC expression on the cell surface. Cells were transfected using the Lipofectamine reagent (Invitrogen). Western blot analysis was performed following standard procedures.

**Results:** SSA inhibited the growth of human lung adenocarcinoma cells in a dose-dependent manner with IC50 values of 3–5 mM, while sulindac sulfide inhibited growth with IC50 values of 50–60 mM. In addition, SSA induced apoptosis and a G0/G1 arrest, and inhibited the proliferation of...
Implications of Oligomeric Endostatin for Cancer Therapy

The ubiquitin proteasome system is an essential intracellular degradation pathway intrinsic to cellular growth control and therapeutics. Endostatin (ES) is a potent inhibitor of angiogenesis, fully suppressing tumor growth in mouse models. In humans, however, it has first polyarsenic compound found in nature was found to be a more potent inhibitor of Myc-induced B-cell lymphomagenesis by the induction of cellular senescence. We hypothesized that small molecule E6AP inhibitors could be useful for the treatment of HPV-related cancers and malignancies where PML expression is suppressed.

Polyarsenic Adamantane-type Compounds – A Promising New Class of Drugs for the Treatment of Drug-resistant Ovarian Cancers

AsA-S1 showed selectivity for cancer cell lines ranging from 27 to 54. We are currently running safety experiments to determine the anti-proliferative activity by modulating glutathione levels. AsA-S1 surpassed the first polyarsenic compound found in nature was found to be far more potent for the induction of proliferation arrest and cell death in cancer cells compared to arsenic trioxide [1]. AsA has the adamantane-type structure of arsenic trioxide in the solid state in which three of the oxygen atoms have been replaced by methylene groups in a contiguous C2-chiral arrangement.

Background: The prognosis for patients with platinum-resistant epithelial ovarian cancer is poor. Therefore, new therapeutic avenues avoiding known molecular resistance mechanisms are urgently required. Arsenic trioxide (AsA), the first polyarsenic compound found in nature was found to be far more potent for the induction of proliferation arrest and cell death in cancer cells compared to arsenic trioxide [1]. AsA has the adamantane-type structure of arsenic trioxide in the solid state in which three of the oxygen atoms have been replaced by methylene groups in a contiguous C2-chiral arrangement.

Material and Methods: Ovarian cancer cell lines and MRC5 cells were obtained from ATCC. Anti-proliferative activities were determined using MTT assay. Apoptosis was demonstrated by flow cytometry using Annexin-V and PI staining. Caspase-3/7 activity was determined by luminessence assays. Modulations of glutathione levels were achieved by buthionine sulfoxime or glutathione pre-treatments.

Results: With AsA we observed (i) a very strong anti-proliferative activity regardless of ovarian cancer histological subtypes, (ii) the absence of cross-resistance with platinum agents (iii) insensitivity towards the expression of major multidrug resistance proteins, (iv) the induction of the intrinsic apoptotic pathway, and mitochondrial depolarisation and (v) dramatic effects on AsA anti-proliferative activity by modulation of glutathione levels. Interestingly, AsA-S1, a sulfur derivative of AsA, exhibited improved anti-proliferative activities (from 16 to 20 fold) compared to AsA against four histological subtypes of ovarian cancer cell lines (IC50 values for proliferation inhibition ranging from 15 to 185 nM). On these cells, AsA-S1 surpassed the activity of carboplatin by three orders of magnitude. When compared to normal human fibroblasts, AsA-S1 showed cell selectivity to ovarian cancer cell lines ranging from 27 to 54. We are currently running safety experiments to determine the maximal tolerated doses of the compounds before starting tumor xenograft models in mice.

A High Throughput Screening Platform for the Identification of Small Molecule Inhibitors of the E3 Ligase E6AP

Way to screen for the lack of activity in clinical trials could be due to the fact that the soluble monomeric ES produced at acidic pH was employed on these studies.

Conclusion: Our work shows that naturally formed ES oligomers produced in P. pastoris at pH 7.4 should preserve the dimeric structure and perhaps lead to a better therapeutic outcome.

A versatile and robust HTS platform has been designed and validated to identify small molecule inhibitors of E3 ligases. In one of the first examples of an E3 ligase high-throughput screen, we have been able to identify potent inhibitors of E6AP, an E3 ligase capable of marking tumor suppressor proteins for proteasomal degradation.

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Material and Methods: We developed a high throughput functional in vitro ubiquitination assay, utilizing E1 and E2 enzymes in conjunction with E6AP and protein substrates. The ubiquitination of E6AP or E6AP substrate was measured with Alphascreen technology.

Results and Discussion: The AlphaScreen assay was optimized, validated and subsequently miniaturized to 1536-well plate format and used to screen a library of 250,000 lead-like molecules for inhibitors of E6AP activity. Additional in vitro E6 ligase assays were also developed and used in both selectivity and counter screens to deconvolute E6AP inhibitors from those that blocked E1 and E2 ligases. We identified a number of compounds with selective inhibitory activity against E6AP.

Conclusions: E6AP is an emerging target for the development of new drugs for the treatment of HPV-related cancers and malignancies where PML expression is suppressed. A versatile and robust HTS platform has been designed and validated to identify small molecule inhibitors of E3 ligases. In one of the first examples of an E3 ligase high-throughput screen, we have been able to identify potent inhibitors of E6AP, an E3 ligase capable of marking tumor suppressor proteins for proteasomal degradation.

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Both Pancreatic Cancer and Pancreatic Stellate Cells Express Novel Anti-angiogenic MiRNAs Reduce in Vivo Tumor Growth

Background: Tumours of the pancreas, which display the most prominent desmoplastic reaction of all epithelial tumours, have been shown to express γ-glutamyl transferase (γGT) at different levels, while soft tissue tumours tend not to express this enzyme [1]. The desmoplastic reaction is mediated by the cancer-associated stellate cells (CA-PSC) that we have found to have expressed up to 30-fold higher levels of γGT on their surface than normal pancreatic stellate cells. GSAO, is an arsenic-based angiogenesis inhibitor [2] currently being tested in a Phase I/IIa clinical trial in adults with solid tumours refractory to standard therapy. GSAO is a pro-drug activated at the cellsurface by-γGT [3].

Material and Methods: Pancreatic cancer cell lines and endothelial cells were obtained from ATCC and Cell Application, respectively. Anti-proliferative activities were determined using MTT and WST-1 assays. CA-PSCs were isolated as described by Völaufen et al. [4]. Subcutaneous murine models of pancreatic cancer expressing various levels of γGT were performed in Balb/c nude mice. GSAO was administered subcutaneously using micro osmotic pumps.

Results: We demonstrated (i) that both pancreatic cancer and CA-PSC cells that express high levels of γGT can transform GSAO into its active form and then induce proliferation arrest and apoptosis of the nearby endothelial cells and (ii) that cancer cell expression of γGT positively correlates with the anti-tumour efficacy of GSAO in two mouse models of pancreatic cancer using γGT-transfected cells or cells naturally expressing high and basal levels of this enzyme. The anti-tumor efficacy of systemically administered GSAO is currently being evaluated in a mouse model of human pancreatic cancer where cancer cells are implanted without or with CA-PSC cells.

Conclusions: These observations suggest that the high expression of γGT on both pancreatic cancer and cancer-associated stellate cells may be employed to deliver a γGT-activated prodrug to the tumour mass. Should the hypothesis prove correct, the γGT status of tumours (by biopsy) will be used as a predictive marker to select patients for Phase II trials who are more likely to benefit from GSAO treatment.

Reference(s)


ABTL0812, a Dual Inhibitor of mTOR and Dihydrofolate Reductase With High Oral Efficacy and Safety Margin in Human Lung and Pancreatic Cancer Xenograft in Mice

Introduction: Most cancer types are the result of multiple gene mutations and/or impairment of several pathways that control cell proliferation and viability. A compound targeting several key pathways may have enhanced efficacy. We describe ABTL0812, which has oral anticancer properties through a novel mechanism of action involving mTOR (mammalian target of rapamycin) pathway and dihydrofolate reductase (DHFR).

Material and Methods: Cellular assays were performed in the A549 lung adenocarcinoma, and Panc-1 and MiaPaca2 pancreatic carcinoma cell lines. Cell proliferation assays were carried out by monitoring bromodeoxyuridine (BrdU) incorporation, cell viability by MTT assay, and protein expression by immunoblotting. Cellular apoptosis was assessed by nuclear staining; and autophagy by LC3-II lipidation assay. In vivo efficacy was studied in nude mice bearing A549 and MiaPaca2 xenografts, in which ABTL0812 was orally administered daily. Body weight and tumor volume were regularly measured. At sacrifice, histopathological analysis of tumors was performed by HE staining. ABTL0812 plasma levels were determined by LC-MS followed by a PKPD analysis.

Results and Discussion: ABTL0812 showed anti-proliferative (cell count and cell incorporation) and cytotoxic (MTT) effects. ABTL0812 abolished DHFR expression, and cell viability was partially rescued by folic acid. ABTL0812 inhibited both mTOR complex 1 and 2 activities, as shown by pS6 (mTORC1) and pS473-Akt (mTORC2) phosphorylation. ABTL0812 did not induce apoptosis (caspase 3 activity and PARP immunoblotting). Furthermore, ABTL0812 cell death was not reverted by pretreatment with caspase inhibitor Z-VAD, and no nuclear staining indicative of apoptosis was observed. By contrast, ABTL0812 treatment induced LC3-II, and necrostatin partially reversed ABTL0812-induced cell death, suggesting a major role for autophagy. ABTL0812 significantly reduced tumor volumes similarly to reference compounds such as docetaxel, erlotinib or gemcitabine. However its impact on body weight was marginal without systemic toxicity. Histopathological analysis of the tumors showed less miltic cells, increased necrotic area, fibrotic cap and inflammatory cells which could contribute to the anticancer effect. Plasma concentrations correlated with tumor volume and in vivo E50 = 955 nmol.

Conclusion: ABTL0812 shows a high anticancer efficacy and safety in human lung and pancreatic cancer xenografts in mice. ABTL0812 induces autophagic cell death by inhibiting mTOR activity and DHFR expression, which makes it a very unique compound. ABTL0812 is currently in preclinical development for lung and pancreatic cancer.

Novel Anti-angiogenic MiRNAs Reduce in Vivo Tumor Growth in Mouse Models

Introduction: Tumours of the pancreas, which display the most prominent desmoplastic reaction of all epithelial tumours, have been shown to express γ-glutamyl transferase (γGT) at different levels, while soft tissue tumours tend not to express this enzyme [1]. The desmoplastic reaction is mediated by the cancer-associated stellate cells (CA-PSC) that we have found to have expressed up to 30-fold higher levels of γGT on their surface than normal pancreatic stellate cells. GSAO, is an arsenic-based angiogenesis inhibitor [2] currently being tested in a Phase I/IIa clinical trial in adults with solid tumours refractory to standard therapy. GSAO is a pro-drug activated at the cellsurface by-γGT [3].

Material and Methods: Pancreatic cancer cell lines and endothelial cells were obtained from ATCC and Cell Application, respectively. Anti-proliferative activities were determined using MTT and WST-1 assays. CA-PSCs were isolated as described by Völaufen et al. [4]. Subcutaneous murine models of pancreatic cancer expressing various levels of γGT were performed in Balb/c nude mice. GSAO was administered subcutaneously using micro osmotic pumps.

Results: We demonstrated (i) that both pancreatic cancer and CA-PSC cells that express high levels of γGT can transform GSAO into its active form and then induce proliferation arrest and apoptosis of the nearby endothelial cells and (ii) that cancer cell expression of γGT positively correlates with the anti-tumour efficacy of GSAO in two mouse models of pancreatic cancer using γGT-transfected cells or cells naturally expressing high and basal levels of this enzyme. The anti-tumor efficacy of systemically administered GSAO is currently being evaluated in a mouse model of human pancreatic cancer where cancer cells are implanted without or with CA-PSC cells.

Conclusions: These observations suggest that the high expression of γGT on both pancreatic cancer and cancer-associated stellate cells may be employed to deliver a γGT-activated prodrug to the tumour mass. Should the hypothesis prove correct, the γGT status of tumours (by biopsy) will be used as a predictive marker to select patients for Phase II trials who are more likely to benefit from GSAO treatment.

Reference(s)

However, the low frequency of BRCA1/2 mutations limits the applicability of this therapeutic strategy. In contrast, alterations in phosphatidylinositol 3-kinase (PI3K) pathway may be more frequent in TNBC. Besides regulating cell metabolism and promoting survival, the PI3K pathway has been also implicated in the maintenance of DNA integrity. In this study we hypothesized that inhibition of PI3K signaling may result in deficient HR with consequent sensitization to PARP inhibition. This phenomenon would occur in BRCA-proficient tumors, providing a therapeutic rationale for treating a larger set of patients with TNBC.

Materials and Methods: PI3K inhibition was attained through either siRNA or treatment with pan-PI3K inhibitors. Clonogeneric, anchorage-independent growth and xenograft assays were used to assess efficacy of treatment. Five patient-derived xenograft (PDX) models were developed by in vivo serial transplantation of tumors from metastatic breast cancer patients after informed consent.

Results: Using BRCA wildtype TNBC cell-lines and PDXs, we demonstrate that PI3K blockade leads to histone-2AX nuclear foci accumulation, a marker of double strand DNA breaks. Concomitantly, inhibition of PI3K suppressed the expression of both BRCA1/2 and Rad51, another component of HR. However, while Rad51 was consistently downregulated in all the animal models, BRCA1/2 levels diminished only in 3 out of 5 PDXs. First assays revealed that BRCA1/2 expression were, at least in part, modulated by the ERK pathway. Indeed, downregulation of BRCA1/2 (both mRNA and protein levels) was prevented by pharmacological MEK blockade. PI3K blockade, achieved by either siRNA of PIK3CA or pharmacological inhibition of the pathway, sensitized BRCA wildtype cell-lines to PARP inhibition in vitro. In vivo, this phenomenon was limited only to those models that presented BRCA1/2 downregulation following PI3K suppression. In these PDXs, simultaneous inhibition of PI3K and PARP resulted in profound anti-tumor response, whereas no superior activity versus single agent was observed in the PDXs where BRCA expression was retained upon PI3K blockade.

Conclusions: PI3K blockade sensitizes to PARP inhibition by promoting BRCA-deficiency that may be regulated by ERK feedback signaling. This combinatorial strategy broadens the applicability of PARP inhibition to BRCA-proficient patients and deserves further investigation to identify accurate predictive biomarkers.

**513 Efficiency and Transport of Taxanes in Human Cancer Cell Lines and Solid Tumors**

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Introduction: Taxanes are successfully used in therapy of breast and ovarian carcinomas. However, inherited or acquired drug resistance of tumor cells to taxanes presents one of the major obstacles in successful therapy. Drug resistance is a multifactorial process that may be related to drug transport, metabolism or alterations in apoptosis induction by taxanes. We explored transport of classical and novel taxanes in cell lines and expression profile of ATP-Binding Cassette (ABC) transporters, caspases and cytokinesis-modulating genes in breast and ovarian carcinomas in order to reveal biomarkers of prognosis and/or prediction of outcome of taxane-based therapy.

Material and Methods: Transport of taxanes (paclitaxel and fluorinated SBT-F taxanes) in taxane-sensitive MDA-MB-435 and resistant NC/ADR-RES cells was measured by HPLC analysis or liquid scintillation. Gene expression was evaluated by real-time PCR with relative quantification and assessed by reverse transcription. Immunofluorescence was used to detect localization of taxanes in tumor cells. Cell proliferation was determined by the MTT assay, and death and verify a possible synergistic effect.

Results and Discussion: Both in cell lines and primary MCL cells, sorafenib induces rapid dephosphorylation of the BCR-associated tyrosine kinases, SYK and Lyn, as well as FAK, a downstream SRC target related with focal adhesion. In line with this, we demonstrate a strong synergy when combining sorafenib with the SYK inhibitor, R406. In parallel, we show that sorafenib also blocks Mel-1 and cyclin D1 translation, which promotes an imbalance between pro- and anti-apoptotic proteins and facilitates the release of Bax from cycin D1, leading to the induction of the mitochondrial apoptotic pathway and caspase-dependent and independent mechanisms. Moreover, sorafenib inhibits MCL cell migration as well as actin polymerization in response to CXCL12. FAK knockdown partially prevents this inhibitory effect, indicating that FAK is a relevant target for sorafenib action in MCL cells. Importantly, this compound resensitizes MCL cells cultured with stroma to bortezomib-induced apoptosis.

Conclusion: Here, we provide first evidence on the molecular mechanism of action of the multikinase inhibitor sorafenib in MCL. We propose that this compound inhibits cell migration and stroma-mediated bortezomib resistance by interfering BCR signaling and protein translation. All these results suggest that sorafenib, alone or in combination with bortezomib-based therapies, may represent a promising approach to treat MCL patients.

**1046 Sorafenib Inhibits Cell Migration and Stroma-mediated Bortezomib Resistance by Interfering BCR Signaling and Protein Translation in Mantle Cell Lymphoma**

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Introduction: Mantle cell lymphoma (MCL) is an incurable B-cell neoplasm harboring the t(11;14)(q13;32) translocation which leads to the overexpression of cyclin D1, with the consequent cell cycle deregulation. Typically, MCL is characterized by bad prognosis and an aggressive course of the disease while current therapies have shown limited efficacy. Thus, our purpose was to evaluate the antitumor properties of the multikinase inhibitor sorafenib in MCL.

Materials and Methods: The sensitivity to sorafenib was analyzed in 9 MCL cell lines and 17 primary MCL cells by flow cytometry. Sorafenib anti-tumor signaling was characterized by quantitative reverse transcription PCR, western blot, immunofluorescence and protein immunoprecipitation. Cell migration analysis was done by counting cells with a flow cytometer, actin polymerization assays and siRNA-mediated knockdown of FAK. Microenvironment was simulated with coculture of MCL cells with follicular dendritic and bone marrow-derived stroma cells.

Results and Discussion: Both in cell lines and primary MCL cells, sorafenib induces rapid dephosphorylation of the BCR-associated tyrosine kinases, SYK and Lyn, as well as FAK, a downstream SRC target related with focal adhesion. In line with this, we demonstrate a strong synergy when combining sorafenib with the SYK inhibitor, R406. In parallel, we show that sorafenib also blocks Mel-1 and cyclin D1 translation, which promotes an imbalance between pro- and anti-apoptotic proteins and facilitates the release of Bax from cycin D1, leading to the induction of the mitochondrial apoptotic pathway and caspase-dependent and independent mechanisms. Moreover, sorafenib inhibits MCL cell migration as well as actin polymerization in response to CXCL12. FAK knockdown partially prevents this inhibitory effect, indicating that FAK is a relevant target for sorafenib action in MCL cells. Importantly, this compound resensitizes MCL cells cultured with stroma to bortezomib-induced apoptosis.

Conclusion: Here, we provide first evidence on the molecular mechanism of action of the multikinase inhibitor sorafenib in MCL. We propose that this compound inhibits cell migration and stroma-mediated bortezomib resistance by interfering BCR signaling and protein translation. All these results suggest that sorafenib, alone or in combination with bortezomib-based therapies, may represent a promising approach to treat MCL patients.

**1047 Joining ROS Against Cancer – Does Vitamin C Improve Photodynamic Therapy Outcome?**

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Background: Photodynamic therapy is a therapy widely investigated as anti-cancer modality. It requires a photosensitizer (PS), a molecule able to absorb light in long wavelengths, appropriate for tissue penetration, and highly selective for tumor tissue. Upon irradiation with visible light, the activated PS provokes a reactive cellular species (ROS) that is responsible to induce cell death. Ascorbic acid (AA), also known as ascorbate or vitamin C, is known for its antioxidant properties. However in supraphysiological concentrations it becomes a prooxidant molecule that might be useful in a therapeutic context. Since both PDT and AA induce the generation of ROS we figured that the combination of these two procedures should enhance cell death. So the aim of this work is to evaluate the outcome of the combination of AA and PDT, using 5,15-bis-(2-bromophenyl)cinnolvin at PS, within cell behavior and death and verify a possible synergistic effect.

Material and Methods: To perform cytotoxicity evaluation, the human cell lines of colorectal adenocarcinoma, WiDr, and esophageal carcinoma, OE19, were incubated with different concentrations of AA for 1 and 4 hours, and the medium was replaced. Cell proliferation was determined by the MTT assay, 24 and 48 hours after incubation. Once established the dose-response curves and determined the IC50 for AA, we established a fixed concentration of 1/4
and 1/8 of that value. Cell cultures were submitted to AA in the referred concentrations in combination with several concentrations of 5,15-bis(2-bromo-3-hydroxyphenyl)chlorin, ranging from 4 × 10⁻⁷ to 5 μM. Cells were irradiated until a total of 10J, and once again cell proliferation was determined by the MTT assay after 24 and 48 hours. To verify intrinsic toxicity of the PS the same protocol was performed without irradiation.

Results: Cell proliferation studies showed a higher cytotoxicity of AA for an exposure of 4 hours with an evaluation at 24 hours (IC₅₀ = 19.5 mM for OE19 cell line, IC₅₀ = 4.36 mM for WiDr cell line). Within combined therapies, referring to OE19 cell line, higher photodynamic effect was obtained with evaluation at 24 hours following exposure to 1/4 of the value of IC₅₀ obtained for AA during 4 hours (IC₅₀ of PS = 2.4 mM). For WiDr cell line, the best outcome was achieved with exposure of one eight of the IC₅₀ of AA during 1 hour with evaluation at 48 hours (IC₅₀ of PS = 5.3 mM). In the studies where irradiation step was omitted it was not possible to reach IC₅₀ even for the highest concentration of PS tested.

Conclusion: The results obtained express a concentration and time dependency of the 5,15-bis(2-bromo-3-hydroxyphenyl)chlorin effect in the presence of AA, not presenting significant differences within different AA exposure times. Although the conclusions are preliminary, we can say that the association of ascorbic acid and photodynamic therapy improves therapeutic outcome.

**[1048] Gene Silencing of β-catenin Inhibits Growth of Wnt/β-catenin Pathway-Dependent Colon Cancer Cell Lines**

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Material and Methods: Wnt/β-catenin pathway is deregulated in many cancer types. Oncogenic mutations of β-catenin (CTNNB1) or inactivating mutations of the APC tumor suppressor gene are related to enhancing cell proliferation and developing various malignancies. The purpose of the present study was to investigate effects of β-catenin small interfering RNA mediated gene silencing on human colon cancer cells with Wnt/β-catenin mutations and examine the β-catenin nuclear translocation in a self-developed reporter cell line.

Material and Methods: Human colon cancer cell lines with CTNNB1 mutation SNU407, SNU1047 and HCT116 were treated with siRNA against β-catenin. Time dependent silencing effects were determined by Western Blot and Real-time PCR analysis. Nuclear β-catenin accumulation was measured using the TOP flash-based 7TFP stable reporter cell line – H1703 and antiproliferative activity was investigated by ATPhit assay.

Results: We showed that siRNA mediated knockdown of β-catenin in colon cancer cell line with constitutively active Wnt/β-catenin pathway provoked decrease of β-catenin nuclear accumulation in a dose dependent manner. After 72h of siRNA treatment we achieved up to 95% inhibition of TCF mediated reporter gene transcription. β-catenin gene silencing inhibits colon cancer cell lines growth in time dependent manner. The antiproliferative effect of examined cell lines extended within 9 days of siRNA treatment. In case of SNU407 the growth inhibition reached 80%.

Conclusion: Interfering with RNAs directed against β-catenin inhibit the canonical Wnt signaling pathway and reduce cell growth of colon cancer cell lines with CTNNB1 mutations. SiRNA mediated β-catenin silencing is promising opportunity for therapy of Wnt/β-catenin signaling dependent colorectal cancer.

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**[1049] A Biodistribution Study of Polyglutamic Polyethyleneglycol Nanocapsules Intended for the Lymphatic Targeting of Anticancer Drugs**

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Background: Lymphatic system is an important route for metastatic spread. Our laboratory is currently involved in the design of new nanomedicines with high biodistribution for the lymphatic system. Previously, we have shown that polyglutamic-polyethyleneglycol coated nanocapsules (PGA-PEG NCs) can load a variety of anticancer drugs and prolong their presence in plasma. The goal of this study was to analyze the biodistribution of PGA-PEG NCs as a new potential lymphophagocyte carrier platform after administration by two different routes: subcutaneously and intravenously.

Material and Methods: PGA-PEG NCs were prepared by modified solvent displacement technique. For labeling, the fluorochrome DiD was introduced in the organic phase during the PGA-PEG NCs preparation process. In vivo biodistribution studies were carried out by injecting labeled PGA-PEG NCs to immunodeficient SCID mice by two administration routes: intravenous (IV) in the tail vein, and subcutaneous (SC) in the interscapular region. The biodistribution was performed after necropsy using an In Vivo Imaging System (IVIS® Spectrum, Caliper Life Sciences) at different time points (6, 24 and 48 hours).

Results: Photon correlation spectroscopy analysis indicated that PGA-PEG NCs are a monomodal particle population with a very narrow distribution of particle sizes centered around 200 nm. PGA-PEG NCs showed a negative zeta potential close to −20 mV, a relatively neutral value that is indicative of the PEGylated surface of the nanocarriers.

The biodistribution studies showed that 6 h after IV administration, PGA-PEG nanocarriers were preferentially accumulated in liver, with some distribution also to lungs, heart and spleen. This pattern was repeated at 24 h and 48 h. At 6 h after SC administration, PGA-PEG NCs accumulated in axillary nodes due to the proximity of the injection site. At 24 h and 48 h after SC administration, the nanocarriers had already reached the cervical and mediastinal lymph nodes. Heart, lungs and liver showed low signal levels. For both administration routes, loss of signal started to be apparent 48 h after the administration of the nanocarriers.

Conclusions: Overall, our results indicate the potential interest of subcutaneous administration of PGA-PEG NCs to induce high, long-lasting accumulation of anticancer drugs in the lymphatics.

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**[1050] Arginine Depletion – Achilles Heel in Gastric Cancer**

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Introduction: Gastric cancer ranks fifth in terms of incidence, and is the fourth most common cause of cancer-related death in Europe. Despite surgical resection together with the preoperative chemotherapy provide promising treatment to the localized disease, the survival rate over the past 20 years has yet to be improved. It is therefore of paramount importance to develop new therapeutic strategies for gastric cancer.

Several types of cancer cells were found to have an elevated requirement for arginine, and therapeutic enzymes based on arginine depletion have been developed. Arginine-depleting enzymes, such as arginase and arginine deiminase (ADI), are now in phase III and phase II clinical trials for hepatocellular carcinoma, respectively. Besides, ADI is also in different stages of clinical trial for melanoma, small cell lung cancer and mesothelioma but its efficacy is limited to cancer cells with downregulated expression of argininosuccinate synthase (ASS) where the recycling of its product, citrulline, from the enzymatic reaction is hampered, which results in the growth inhibition of cancer cells. However, cancer cells with expression of ASS are insensitive to ADI treatment. Arginase converts arginine to ornithine where expression of the ornithine transcarbamylase (OTC) is down-regulated. These cells are unable to recycle the ornithine even when ASS and ASL are expressed, thus resulting in growth inhibition. Here, through a series of in vitro studies, we explored the possibilities of using arginine-depleting enzyme as a therapeutic strategy for gastric cancer treatment.

Materials and Methods: A panel of gastric cell lines (MKN45, BCG823 and AGS) was exposed to the arginine-depleting enzymes and the anti-proliferative effects were determined using MTT cell viability assay. The expression profiles of the urea cycle enzymes were analyzed using RT-PCR and immunoblot which provide information to explain the observed cellular responses. The impacts of arginine depletion on cell cycle distribution and cell death mechanisms were analyzed by flow cytometry.

Results and Discussion: ADI is ineffective to all of the gastric cancer cell lines possibly due to the expression of ASS which converts citrulline back to arginine. Arginase exhibits a potent growth inhibition for all the cell lines tested. Downregulation of OTC prevents recycling of ornithine, and arrest the cancer cells at S phase. Treatment by arginase also triggers apoptotic cell death in MKN45 in a time-dependent and dose-dependent manner. Further studies show that the apoptosis is caspase-dependent and possibly regulated by the mitochondrial apoptotic pathway in both MKN45 and BCG823.

Conclusion: Preliminary data suggest that arginase exerts a potent growth inhibition on gastric cancer cell lines through cell cycle arrest and inducing apoptosis in MKN45 and BCG823. We believe that arginase should be further explored in a pre-clinical setting for its potential as a treatment of gastric cancer.

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**[1051] Knockdown of LINE-1 Enhances Sensitivity to 5-FU in LINE-1-hypomethylated Colorectal Cancer Cell**

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Introduction: Epigenetic alterations including hypermethylation of gene promoter regions and global hypomethylation of DNA are common in colorectal cancer (CRC). We recently reported that long interspersed nuclear element 1
Polymersomes are synthetic block co-polymers that self-assemble in water to form membrane-enclosed nanovesicles. Polymersomes have the potential to encapsulate and carry chemotherapeutic drugs into cells thereby reducing the off-target toxicity that often compromises anti-cancer treatment. Here, we assess the in vitro efficiency of polymersomes to encapsulate chemotherapeutic agents for the effective delivery to head and neck squamous cancer cells (HNSCC) cultured as monolayers and as three dimensional tumour spheroids.

**Materials and Methods:** HNSCC were grown as monolayers or as multi-cellular tumour spheroids (MCTS), to model an expanding tumour island. Polymersomes loaded with rhodamine were internalised by HNSCC within 10 minutes of administration and maximal delivery was achieved within 30 minutes. When delivered to MCTS, polymersomes were internalised by over 80% of cells within 120 hours and importantly are shown to penetrate into hypoxic regions. Both doxorubicin and paclitaxel were loaded into polymersomes with high encapsulation efficiencies. Furthermore, both drugs can be dually loaded to enable combinational delivery. Loaded polymersomes demonstrated similar killing of HNSCC grown as monolayers compared to the same concentration of free drug. When delivered to MCTS, loaded polymersomes caused growth inhibition and extensive damage of the tumour spheroids. Current work involves the study of the cellular uptake mechanisms involved in the internalisation of the polymersomes to further the knowledge of this novel drug delivery system.

**Conclusion:** Polymersomes offer a novel drug delivery system for the effective delivery of chemotherapeutics for the treatment of head and neck cancer.

**[1052] Polymersomes – a Novel Drug Delivery System for the Delivery of Chemotherapeutic Agents to Oral Cancer Cells**

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**Introduction:** Polymers are synthetic block co-polymers and have been shown to encapsulate chemotherapeutic agents for the effective delivery to head and neck squamous cancer cells (HNSCC) cultured as monolayers and as three dimensional tumour spheroids.

**Materials and Methods:** HNSCC were grown as monolayers or as multi-cellular tumour spheroids (MCTS), to model an expanding tumour island. Polymersomes loaded with rhodamine were internalised by HNSCC within 10 minutes of administration and maximal delivery was achieved within 30 minutes. When delivered to MCTS, polymersomes were internalised by over 80% of cells within 120 hours and importantly are shown to penetrate into hypoxic regions. Both doxorubicin and paclitaxel were loaded into polymersomes with high encapsulation efficiencies. Furthermore, both drugs can be dually loaded to enable combinational delivery. Loaded polymersomes demonstrated similar killing of HNSCC grown as monolayers compared to the same concentration of free drug. When delivered to MCTS, loaded polymersomes caused growth inhibition and extensive damage of the tumour spheroids. Current work involves the study of the cellular uptake mechanisms involved in the internalisation of the polymersomes to further the knowledge of this novel drug delivery system.

**Conclusion:** Polymersomes offer a novel drug delivery system for the effective delivery of chemotherapeutics for the treatment of head and neck cancer.

**[1053] New Strategies to Inhibit Tumor Cell Proliferation and Tumor Metastasis by S100P Monoclonal Antibodies**

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**Background:** Metastasis represents the most devastating stage of malignancy and the leading death cause of cancer. An accurate description of the cellular and molecular mechanisms that drive this metastatic process is of utmost importance to facilitate the rational development of therapies that effectively allow metastatic disease to be controlled and treated.

**In the last years, intensive research in this field has shed light on some molecular targets as the novel metastatic factor S100P protein and its promising role as a key player in metastasis formation and poor clinical outcome. The identification and functional characterization of specific regulators of S100P might be exploited for therapeutic applications.**

**Materials and Methods:** Monoclonal antibodies able to neutralize the extracellular activity of S100P were obtained in our lab as therapeutic agents. In order to better elucidate the pivotal role of S100P in tumor cell proliferation and survival, we used either blocking mAbs or S100P knockdown (by RNA interference) in vitro. From the suggested mechanism that the S100P expression has a role in S100P activity, we expected that the knockdown of S100P suppressed the cytotoxic activity of S100P. Contrary to our expectations, the knockdown of S100P did not suppress but rather enhanced the activity of S100P. The study provided the novel strategy to enhance the effect of S100P activity and to investigate whether the increased expression of S100P by S100P is directly related to the S100P expression in the CRC cell with S100P hypomethylation because suppression of S100P is a possible strategy to enhance the activity of 5-FU.

**Results and Discussion:** S100P methylation level in tumor, a surrogate of global methylation, predicts prognosis and response to oral fluoropyrimidines of CRC patients. In this study, the mechanism underlying the association between LINE-1 methylation and sensitivity to 5-FU was investigated.

**Material and Method:** CRC cells were used to analyze LINE-1 methylation level, LINE-1 mRNA expression and sensitivity to 5-FU by quantitative methylation specific PCR, Northern blotting and colony forming assay, respectively. Expression of phospho-histone H2AX was analyzed by Western immuno-blotting. RNA interference was employed to knockdown LINE-1 expression.

**Results and Discussion:** LINE-1 methylation level in CRC cells was correlated with LINE-1 mRNA expression, in which hypomethylated CRC cell expressed high level of LINE-1 mRNA. The LINE-1-hypomethylated CRC cell also showed higher sensitivity to 5-FU. LINE-1 mRNA expression was increased after 5FU exposure in the cells showing LINE-1 hypomethylation, which is associated with upregulation of phospho-histone H2AX, a marker of DNA double strand break (DSB). These results suggest that 5FU action to induce DSB and exert cytotoxic effect is mediated, in part, by augmentation of LINE-1 expression in the CRC cell with LINE-1 hypomethylation because LINE-1 gene encodes endonuclease, a possible player to induce DSB. To investigate whether the increased expression of LINE-1 by 5-FU is directly involved in the 5-FU action, LINE-1 expressing CRC cell was treated with 5-FU following the knockdown of LINE-1 by RNA interference. From the suggested mechanism that the LINE-1 expression has a role in 5-FU action, we expected that the knockdown of LINE-1 suppressed the cytotoxic effect of 5-FU. Contrary to our expectations, the knockdown of LINE-1 did not suppress but rather enhanced the activity of 5-FU. The study provided the novel strategy to enhance the effect of 5-FU although it did not reveal the mechanism underlying the association between LINE-1 methylation and sensitivity to 5-FU.

**Conclusion:** The LINE-1 expression itself has no significant role in the association between LINE-1 methylation level and the sensitivity to 5-FU. Suppression of LINE-1 is a possible strategy to enhance the activity of 5-FU in CRC with LINE-1 hypomethylation.

**[1054] Evaluation of Hypoxia Inducible Factor-1-Alpha (HIF1A) After Treatment With Melatonin in Breast Cancer Cell Line**

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**Introduction:** Breast cancer can cause death in patients due to tumor growth and metastasis development. The hypoxia could limit tumor angiogenesis preventing its growth and progression however, tumor cells induce the transcription of HIF1A that promotes angiogenesis. Several researches focus on developing new therapeutic agents to inhibit angiogenesis factors, such as melatonin, a hormone secreted by the pineal gland. The aim of this study was to evaluate the change in HIF1A protein expression in breast cancer cell line, after the induction of hypoxia and treatment with melatonin.

**Material and Method:** MDA-MB-231 cell line was cultured in DMEM (high glucose) and incubated at 37°C in 5% CO2. At first, the cells were divided into 7 groups to measure the cell viability by MTT assay: Group I: no treatment (Control), Group II received 100µM of CoCl2, hypoxia, and Groups III to VII also received CoCl2 and different concentrations of melatonin (0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM). Once established, the association of melatonin, the cells were divided in 3 groups to assess the expression of HIF1A by immunocytochemistry and Real-Time PCR. Group A (control), Group B with only CoCl2 and Group C with CoCl2 and 1µM of melatonin. The quantification of immunexpression was performed by optical densitometry (ImageJ software), demonstrating mean optical density (MOD). The results were compared by ANOVA, followed by the Bonferroni test.

**Results and Discussion:** The results showed that all groups treated with melatonin under hypoxia conditions had reduced cell viability. The concentration of 1 µM showed decrease of 48.13%, being considered the optimal concentration of melatonin for treatment in vitro. By immunocytochemistry,
there was significant difference between the HIF1α expression in all groups (p < 0.0001). The expression of HIF1α was lower in the control group (MOD = 114.2) when compared to the other groups (p = 0.001).

Conclusion: Our results suggest that melatonin is effective in control tumor angiogenesis and can be a possible therapeutic alternative for breast cancer.

1055 Bacterial Protein Azurin as a New Candidate Drug to Treat P-cadherin Overexpressing Breast Cancer

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P-cadherin overexpression occurs in about 30% of all breast carcinomas, being an important prognostic factor for worse patient survival (Paredes et al. 2007; Albergaria et al., 2011). We have previously shown that its expression promotes invasion, motility and migration of breast cancer cells, which is due to the induced secretion of metalloproteases (MMPs) to the extracellular medium and to the shedding of a soluble form of this protein (sp-cad) (Ribeiro et al., 2010). These P-cadherin-overexpressing tumors show a highly aggressive clinical behavior, due to metastasization, and not have until now a targeted therapeutic approach. A water-soluble protein (14kDa), produced by the induced secretion of metalloproteases (MMPs) to the extracellular medium, angiogenesis and can be a possible therapeutic alternative for breast cancer.

Surface Plasmon Resonance (SPR) experiments revealed strong interactions between azurin and both P- and E-cadherins (k = 2.05 × 10^10 M−1 s−1, m = 1055). In vitro, one single dose of azurin at 50–100 nM caused a specific decrease on P-cadherin protein levels from 30–50% in two different breast cancer cell lines. On the other hand, the levels of E-cadherin, a known tumor suppressor, remain unaltered or even increased. These results were further confirmed by immunofluorescence. Additionally, the sp-cad was reduced after azurin treatment and Matrigel invasion assays demonstrated that azurin reduces the invasive phenotype of the cells, concordant with the decrease on P-cadherin. We have also observed a decrease in MMP2 activity in the extracellular media of azurin treated cells. Moreover, azurin led to a decrease in the phosphorylation levels of both FAK and Src proteins, non-receptor tyrosine kinases activated by P-cadherin overexpression, involved in several signaling pathways that regulate cell-cell and cell-matrix adhesion. Altogether, our data show that azurin exhibits a specific preference for P-cadherin, suggesting that azurin, by inhibition of P-cadherin, may have a potential use in the treatment of breast carcinomas overexpressing this protein.

1056 Synergistic Antitumor Activity of Lapatinib and Retinoids on Breast Cancer Cell Lines: A Novel Subtype of Breast Cancer With Co-amplification of ERBB2 and RARA

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Background: All-trans retinoic acid (ATRA), the only clinically available cyto-differentiating agent, has potential for the therapy/chemoprevention of breast carcinoma. At least five distinct subtypes of breast tumors have been described on the basis of gene expression profiling with the most important determinants of these subtypes being the hormone receptor status (estrogen receptor α, ER, and the progesterone receptor, PR) or the amplification/overexpression of ERBB2/HER2. Indeed, ER and the EGFR family of tyrosine kinase receptors, to which Her2 belongs, have been proven to be crucial triggers of breast cancer proliferation, and currently represent the most effective targeted anticancer therapy. Given the heterogeneous nature of breast cancer, a rational use of ATRA and derivatives (retinoids) in the clinic requires the identification of patients that would benefit from retinoid-based protocols.

Material and Methods: FISH and Q-PCR analysis were used to identify ERBB2 and retinoic acid receptor alpha (RARα) co-amplification in a cohort of 76 patients selected for ERBB2 amplification only. Proliferation (trypan-blue exclusion) and apoptosis ( caspase activity) assays were used to detect viability and cell death in a panel of breast cancer cell lines recapitulating breast cancer heterogeneity. RNA interference was used to functionally validate the role of specific genes found modulated by ATRA and Lapatinib via gene-expression microarray.

Results: 23–32% of the human ERBB2 amplified breast cancers show co-amplification of RARA, encoding the retinoic acid receptor β, RARB. This represents a novel subtype of breast cancer characterized by remarkable sensitivity to ATRA and RARα agonists, regardless of positivity to the estrogen receptor, a known modulator of retinoid sensitivity. In estrogen receptor-negative cellular models showing coamplification of ERBB2 and RARA, simultaneous targeting of the corresponding gene products with combinations of lapatinib and ATRA causes synergistic growth inhibition, cyto-differentiation and apoptosis. The antiproliferative/apoptotic responses triggered by lapatinib+ATRA was found to be modulated by RARRES3 and Foxo3a. Induction of the retinoid-dependent RARRES3 protein by ATRA stabilizes the effect of lapatinib inhibiting ERBB2 phosphorylation (SPD) and co-amplification and activation of the transcription factor FOXO3A integrates ATRA-dependent transcriptional and lapatinib-dependent posttranscriptional signals, controlling the levels of effector proteins like the antiapoptotic factor, BIRC5.
Conclusion: Retinoids and lapatinib act synergistically to induce differentiation and apoptosis in breast cancer cells characterized by the co-activation of ERBB2 and RARA via RARE3 and FoxO3A signaling pathways.

1058 S100A4 Acts Synergistically With VEGF in Promoting Angiogenesis and Neutralizing Monoclonal Antibody Against S100A4 Could Be a Novel Strategy to Combat Solid Tumors
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Background: Angiogenesis is one of the most exciting fields for cancer therapy. Tumor growth and metastasis are dependent on the ability of the tumor cells to induce and maintain neovascularisation of tumor. Angiogenesis requires an orderly activation of genes controlling proliferation, invasion, migration and survival of endothelial cells. At present, VEGF is the best-validated angiogenic factor and a critical mediator of angiogenesis pathway and several approaches are being used to inhibit VEGF function and treat patients with cancer. Advances in the molecular understanding of the angiogenic cascade and tumorigenesis resulted in the identification of the protein S100A4, secreted by tumor and stromal cells, as a key player involved in endothelial cell migration and therefore a promising factor for therapeutic applications interfering tumor angiogenesis and progression.

Materials and Methods: We have developed a neutralizing monoclonal antibody against S100A4 protein. An endothelial cell migration assay was used to study the synergistic effect of S100A4 and VEGF and the monoclonal antibody inhibitory activity.

In addition, a S100A4 knockdown and an overexpressing S100A4 in vivo approach was developed to comprehend the critical role mediated by the protein in tumor angiogenesis and tumor development.

We also tested the therapeutic activity of our S100 monoclonal antibody in a subcutaneous xenograft pancreatic tumor model.

Results: In vitro directed migration assay revealed a synergistic mechanism of action between S100A4 and VEGF on HUVECs, increasing the expression of the VEGFR2 (KDR) in a dose-dependent manner and our S100 mAb blocked this activity.

In experimental tumor model overexpressing S100A4 protein in a melanoma cell line, there was a significant increase of tumor vascularisation and tumour growth.

We also observed a dramatically reduction in tumor growth using stable transfected MiaPACA-2 pancreatic tumor cell line with siRNA against S100A4. Moreover, in vivo specific inhibition of the extracellular S100A4 activity in MiaPACA-2 tumor model by intraperitoneal administration of the mAb, induced a significant suppression in tumor vascularisation and growth delay (p < 0.05).

Conclusion: Taken together all of these results, we have clearly elucidated for the first time a therapeutic strategy blocking the extracellular role played by the S100A4 protein with a first in class monoclonal antibody. This new drug alone or in combination with anti-angiogenic or chemotherapeutic agents could be then a critical inhibitory strategy to decrease tumor vasculature and consequently tumor development.

1060 SUMOylation of FOXM1 Regulates its Activity and Cytotoxic Drug Response
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Background: The forkhead box transcription factor FOXM1 is an essential effector of the G2/M phase transition, mitosis and DNA damage response and is frequently deregulated during tumorigenesis and the development of chemoresistance. Here we report the SUMOylation of FOXM1 which was enhanced during genotoxic drug treatment and mitotic arrest.

Material and Methods: Using a SUMOylation deficient mutant FOXM1 and an Ubch9-fusion directed SUMOylation assays we studied the role of FOXM1 SUMOylation in cancer chemotherapeutic drug treatment.

Results: We showed that SUMOylation of FOXM1 results in its cytoplasmic localisation, and a decrease in transcriptional activity. Moreover, SUMOylation enhanced FOXM1 degradation, and a SUMOylation deficient FOXM1 mutant showed reduced chemotherapeutic drug resistance. Consistently, a SUMOylation deficient FOXM1 mutant enhanced cell proliferation compared to wild type, and fusion of Ubch to wild type FOXM1 slowed time from mitotic entry to exit and resulted in sustained cyclin B1 expression, compared to a SUMOylation deficient FOXM1-Ubch fusion or control.

Conclusions: These data suggest that SUMOylation of FOXM1 during eurubicin and paclitaxel treatment may reduce its activity by enhancing its degradation and cytoplasmic localisation.

[1051] Transgenic Expression of Wild-type or Phosphatase-deficient Mutant PTEN Restores Sensitivity to Resveratrol in PTEN-deficient Ovarian Cancer Cells
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Introduction: Ovarian cancer ranks as the fifth leading cause of cancer-related deaths among women, and the leading cause of death from gynecological cancer. First-line therapy for advanced stage disease includes maximal surgical debulking followed by platinum/taxane chemotherapy with response rates of over 80% (Du Bois et al., 2005). However, most patients will eventually relapse with chemoresistant tumors. Many efforts have been devoted to identify the mechanisms of tumor resistance. Abnormal activation of the Akt pathway has been identified as a main cause for acquired polychemoresistance in ovarian cancers. PTEN is an oncosuppressor with dual lipid- and protein-phosphatase activity. While the substrates of the latter activity are not fully known, the lipid-phosphatase activity antagonizes Akt activation, thus implicating loss of PTEN in chemoresistance. Resveratrol is a naturally occurring food-derived polyphenol with anticanerogenic properties. Resveratrol signals in the cell through inhibition of class I PI3-kinase (which normally activates the Akt pathway) and activation of AMPK, besides other transduction pathways involving SIRT1. Here we tested the ability of resveratrol to kill ovarian cancer cells.

Materials and Methods: OVCA-R3, OV42, SKOV-3 and A2780 ovarian cancer cell lines were cultured under standard culture conditions. Cell death was assessed by annexinV-positivity after treatment for up to 48 h with Resveratrol. The PTEN/Akt pathway was assessed by western blotting. Standard molecular biology protocols were used for cDNA cloning, sequencing and cell transfection.

Results and Discussion: OVCA-R3 cells showed resistance toward Resveratrol (and also to VP16 and doxorubicin) compared to the other cell lines. OVACR-3 were shown to express one wild-type PTEN allele and one phosphatase-deficient PTEN allele. Ectopic expression of either a wild-type or a lipid-phosphatase deficient or a dual-phosphatase-deficient mutant PTEN restored sensitivity to resveratrol.

Conclusion: Independently of its known lipid- and protein-phosphatase activity, PTEN possesses a so-far-unknown activity that renders cancer cells sensitive to anticancer drugs.

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[1062] Selection for Trabectedin Resistance Induces Stable and p53-independent Hypersensitivity Against Cisplatin in Human Colon Cancer Cells
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Introduction: Trabectedin is a marine-derived anticancer compound approved for the treatment of soft tissues sarcoma and ovarian carcinoma. It binds to the minor groove of DNA leading to a unique blockade of selective transcription factor-activated gene transcription. Resistance factors for trabectedin are widely unknown so far. Consequently, we selected isogenic colon cancer cells with p53 wild-type (wt) and knock-out (ko) background against trabectedin and investigated the respective chemosensitivity patterns.

Materials and Methods: Gene expression studies were performed using real-time PCR, Western blot and fluorescence microscopy. Cytotoxic and cytostatic activities were determined by MTT and 3H-thymidine incorporation assays. Cisplatin accumulation was estimated by measuring cellular platinum levels using ICP-MS. DNA damage was determined using the alkaline comet assay. In vivo response to cisplatin (3 mg/kg) was tested in subcutaneous xenograft in SCID mice.

Results: HCT-116 human colon cancer cells (p53 wt) were highly sensitive against trabectedin with an IC50 value in the low nM range. Loss of p53 resulted in even enhanced (1.9-fold) trabectedin sensitivity. Selection against increasing concentrations of trabectedin resulted in both genetic background in cell sublines with low but significant resistance against trabectedin (around 2-fold enhanced IC50 values). When testing for cross-resistance, an unexpected and drastic sensitivity against cisplatin became obvious (10- to 15-fold lowered IC50 values). Interestingly, this hypersensitivity was persistent even following a 6 month culturing without trabectedin. Neither cellular platinum accumulation and DNA-platination nor induction of DNA damage ( Comet assay) was significantly changed in the trabectedin-selected sublines in response to cisplatin. In contrast, massively enhanced DNA damage signal induction including phosphorylation of H2AX in the trabectedin-selected sublines was followed by potent apoptosis execution in vitro. Accordingly, cisplatin treatment was active solely against the trabectedin-selected but not the parental HCT116 cells in xenograft experiments in vivo.
Conclusion: Resistance development of colon cancer cells against trabectedin renders them persistently hypersensitive against platinum drugs. This suggests that trabectedin might be included in combination regimens and chemotherapeutic regimens to counteract acquired resistance development.

**1053** Loss of Heterozygosity (LOH) of Single Nucleotide Polymorphisms in DNA Repair Genes Associated With Cisplatin Resistance in Osteosarcoma

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**Background:** Cisplatin (CDPP) is one of the most frequently used drugs for osteosarcoma chemotherapy. Since acquired cellular resistance to CDPP could be associated with increased gene-specific DNA repair efficiency [Zhen et al., 1992] we have evaluated the Loss of Heterozygosity (LOH) of specific DNA repair polymorphisms as possible cause for increased DNA repair efficiency in osteosarcoma cells, in a recent pharmacogenetic study [Biason et al., 2011] we have investigated the clinical effect of DNA repair gene polymorphisms (ERCC1, ERCC2, ERCC5) in a series of 130 high-grade osteosarcoma patients homogeneously treated neoadjuvant cisplatin-based therapy. We founded that the wild type genotype of ERCC2 rs1797973 was significantly correlated with a worse clinical outcome in terms of event free survival, probably because of a more efficient DNA repair activity that reduce CDDP cytotoxicity.

**Materials and Methods:** We have genotyped osteosarcoma cell lines sensitive to CDPP, methotrexate and doxorubicin (U-2OS, Saos-2), and variants of the same cell lines which were resistant to increasing concentration of all the three drugs. The single nucleotide polymorphisms (SNPs) of XPD (rs13181 and rs1799793), XPG (rs17655), and ERCC1 (rs2312986 and rs11615) were analysed by TaqMan genotyping assays.

**Results:** Although variants resistant to methotrexate or doxorubicin did not show LOH of any SNP analysed, CDPP-resistant cell lines showed several LOHs. In particular, LOH for the variant SNP rs1799793 (23591G>A, Asp312Asn) in the ERCC2 gene was found in the two U-2OS variants with highest CDPP resistant levels resulting in homozgyosity for wild type rs1797973 of ERCC2. In addition, LOH of the normal SNP rs13181 of ERCC2 was found in these two CDPP-resistant variants leading to homozgyosity of the variant allele.

**Conclusion:** Our results indicated that in human osteosarcoma cell lines the restoration of the complete efficiency of DNA repair was associated with tumour resistance to CDPP. This lead to the suggestion that in vivo resistance to therapy regimens containing CDPP might be associated with development of LOH of ERCC2 in these tumours. To identify the addiction effect of LOH of the two ERCC2 variants on CDPP resistance in osteosarcoma functional studies are ongoing.

**1054** Investigation of Some Small Heterocyclic Compounds as Potential Antitumor Agents

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**Background:** The search for new antitumor compounds is the imperative for Organic Chemistry, Beograd, Serbia in their action to tumor cells in comparison to normal immunocompetent cells. Tetraxane (5) showed low activity against treated tumor cells. Concentrations inducing 50% decrease in tumor and peripheral blood mononuclear cells survival (IC50) of compounds 2 and 3 in their action to tumor cells in comparison to normal immunocompetent cells, regardless of their proliferating status. The presence of fluorine substituent (compound 3) reduces cytotoxic potential, but increase the selectivity in the antitumor action of investigated 4-aminooquinolines.

**Conclusions:** Results obtained showed that investigated 4-aminooquinolines, are candidates for further analyses on experimental animals, in vivo.

**IC50** Values for the 72 h of action of investigated SHC determined by MTT test

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC50 [nM]</th>
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<tbody>
<tr>
<td>HeLa</td>
<td>5.3 17.2 25.0 49.1 75.0</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>5.2 16.2 24.0 46.6 65.3</td>
</tr>
<tr>
<td>MCF7</td>
<td>6.0 22.5 33.9 35.6 78.3</td>
</tr>
<tr>
<td>K562</td>
<td>6.3 25.8 35.9 50.1 75.1</td>
</tr>
<tr>
<td>PBMc-PHA</td>
<td>6.0 45.8 70.6 53.0 &gt;80</td>
</tr>
<tr>
<td>PBMc-PH4</td>
<td>6.3 36.6 59.2 38.8 &gt;80</td>
</tr>
</tbody>
</table>

**1055** Studies on the Growth Inhibitory and Anti-Invasive Properties of a “Comb-molecule” Designed to Target EGFR and C-Src

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**Background:** In many solid tumors, including breast, lung and prostate carcinomas, it has been shown that c-Src synergizes with the epidermal growth factor receptor (EGFR) to promote cell proliferation and invasion. While molecules with EGFR and c-Src targeting function have been designed, produgs capable of releasing optimized inhibitors of the two targets are scant.

**Materials and Methods:** We designed and synthesized AL776, which contains a quinazoline head (AL621) targeted to EGFR and a hydrolysable linker that connects it to dasatinib (Sprycel®), a clinically approved c-Src inhibitor). The growth inhibition of AL776 was assessed in a panel of isogenic NIH3T3 cell lines (wt, Neu/ErbB2 and Her14/EGFR transfected) using the SRB assay. Wound healing and motility assays were performed on 4T1 mouse and MDA-MB-231 human breast cancer cells. Additionally, Boyden chamber invasion assays was performed on the NIH3T3 transfectected cells and the breast cancer cells. In all assays, the potency of the combi-molecule was compared with that of gefitinib (Iressa®), a clinically approved EGFR inhibitor) and dasatinib.

**Results:** AL776 exhibited stronger EGFR inhibitory potency than dasatinib with an IC50 in the submicromolar range (0.12 uM) and a stronger c-Src inhibitory effect than gefitinib with subnanomolar potency (IC50 = 0.12 nM). Studies in NIH3T3 cells transfectected with EGFR or Neu showed that: (a) AL776 was like gefitinib and dasatinib, extremely selective for the transfectants, (b) it showed 2-3-fold greater growth inhibitory potency than gefitinib or dasatinib, two optimized drugs for their respective target, (c) it recapitulated the growth inhibitory activity of an equimolar mixture of gefitinib+dasatinib. Results from the wound-healing and motility assays showed that AL776, like dasatinib, was capable of blocking migration in 4T1 and MDA-MB-231 cells at nanomolar doses. Boyden chamber invasion assay showed that AL776 blocked invasion of cells at nanomolar doses as well. HPLC analysis of its degradation in mouse plasma demonstrated that it can generate intact dasatinib(AL621).

**Conclusions:** We have successfully achieved the synthesis of a molecule with strong antiangiogenic and anti-invasive properties, which is capable of mimicking the activity profile of two potent clinical molecules. The results in toto suggest that designing molecules to inhibit c-Src and EGFR can be an effective strategy to block aggressive proliferation and invasive properties of solid tumor cells.

**1057** Problems With Rapamycin Alone in Gliona Malignant

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Malignant glioma is the most common primary brain tumor in adult and has a dismal prognosis. The mammalian target of rapamycin (mTOR) plays a central role in regulating the proliferation of cancer cells, and mTOR-specific inhibitors such as rapamycin analogs are considered as a promising therapy for malignant glioma.

In this study, we investigated the possibility of using mTOR inhibitors to treat gliomas. Here molecular marker, phosphorylation of S6 protein was used to monitor biological effects of mTOR inhibitors, and its expression level was found to be decreased more in U87MG glioma after treatment with high dose of rapamycin or its analogue torisel (10 mg/kg or 25 mg/kg) than low dose of rapamycin (3 mg/kg).

Therefore, efficacy study in rodent brain tumor model was done with high doses of rapamycin and torisel, which increased survival time of animals with brain
tumor. Together with the clinical trial data showing that low dose of mTOR inhibitor (3 mg/kg) had no evidence of efficacy in patients with recurrent GBM, it is concluded that systemic administration of rapamycin analogues may not be a treatment option for patients with malignant glioma due to their intolerable high dose, which may result in severe side effects.

Potential, Cellular Inhibitors of Glucose-6-phosphate
Dehydrogenase – Potential for Novel Therapeutic Intervention in Cancer

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Introduction: Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme in the pentose phosphate pathway (PPP) and catalyses the oxidation of glucose-6-phosphate to glucose-6-phosphate dehydrogenase, with the concomitant production of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Alongside an important role in the production of biosynthetic precursors such as ribonucleotides, this pathway is a major cellular source of NADPH. As such, the pathway plays a critical role in both fatty acid & cholesterol biosynthesis (thus supporting cell division) and maintaining glutathione in its reduced state (GSH), ameliorating cellular stress arising from reactive oxygen species (ROS). Given these key roles, modulation of the pathway has potential therapeutic application in cancer, and inhibition of G6PD is an attractive target, particularly in combination with standard of care radiotherapy or cytotoxic drugs which can increase ROS.

Materials and Methods: The steroid nucleus of DHEA was used as a starting point for exploring SAR of novel steroid inhibitors of G6PD. Human G6PD was cloned into a His-tagged vector and expressed in E. coli. An enzyme assay was developed using purified G6PD which measured the amount of NADP reduced to NADPH in the presence of novel inhibitors. Inhibition of G6PD in cells was carried out with HEK293T cells. Novel inhibitors were added to cells together with 6-amidonicotinamide and incubated for 4–6 h. Inhibition of 6-phosphogluconate accumulation was measured, following cell lysis, by mass spectrometry.

Results: Several compounds with approximately 10-fold improved potency over DHEA were identified and this improved activity translated to efficacy in a cellular assay. The novel compounds generally have good physicochemical properties and satisfactory in vitro DMPP parameters.

Conclusion: We have developed a series of novel steroid G6PD inhibitors which, in our hands, modulate flux through the PPP. These compounds may be useful for examining the role of G6PD inhibition in the cell, and will assist the future design of more potent steroid inhibitors with potential therapeutic utility.

Mouse p53-deficient Cancer Models as Platforms to Obtain Genomic Predictors for Human Cancer Clinical Outcome

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Background: Our study aims to validate mouse models with p53-deficiency as tools to develop genomic-based predictors of human cancer clinical outcome.

Material and Methods: Genome-wide gene expression microarray experiments were performed in skin carcinomas arising in p53-deficient transgenic mouse, and compared with microarray experiments from carcinomas performed in various mouse models of breast and lung cancer. Based on these models, and using various interspecies comparison and biostatistical tools, we have obtained genomic predictors of breast cancer and lung adenocarcinoma using training datasets containing microarray analysis and censored survival data of cancer patients. Validation was performed on different published testing datasets and in our cohort of FFPE blocks of tumor samples using qRT-PCR.

Results: Previously, we have demonstrated that the transcriptome of p53-deficient skin carcinoma mouse models is similar to human cancers characterized by TP53-mutation and/or poor clinical outcome. Here we demonstrate that this transcriptome (692-gene signature) is significantly shared by carcinomas of mouse models of breast and lung cancer with p53-inhibition. Furthermore, based on gene expression we have obtained and validated predictor tests for human breast and lung adenocarcinoma clinical outcome.

Conclusions: Our results indicate that p53-pathway deficiency assessed using gene expression from mouse models could accurately stratify human cancer patients and provide new antimetastatic targets for p53-defective tumors.

Analysis of Lipid Composition in Human Cancer Cell Lines Before and After Treatment With Protocolestrogenic Acid Isolated From Cetraria Islandica

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Background: Cancer cells show differences from healthy normal cells in their metabolism, which contribute to their survival and growth. Lipids have numerous functions in biological processes, structural as well as regulatory roles, Hydroxyeicosatetraenoic acids (HETEs), the products of lipoxygenase (LOX) pathways have been implicated in cancer development and protocolestrogenic acid (PA), a secondary lichen metabolite is a potent inhibitor of 5- and 12-LOX, purpose of this work is to develop an HPLC-MS/MS method for evaluation of lipid composition in human cancer cell lines, before and after treatment with PA.

Material and Methods: PA was purified from petroleum ether extract of Cetraria islandica with preparative high-pressure liquid chromatography. A HPLC-MS/MS method was developed and optimized for quantification of HETEs and LTB4, utilizing chemometric approach. Different sample preparation methods were tested and parameters optimized for factorial experimental design. The amount of HETEs in both cultured cancer cells and cultural medium supernatants were analyzed with the optimized HPLC-MS/MS method. Cells were stimulated with calcium ionophore (A23187) and treated with PA.

Results: Preparative HPLC was successfully used for crude-purification to obtain one of the major secondary metabolites in the lichen Cetraria islandica (L.) Ach., PA and its tautomeric lithochenic acid. NMR and analytical HPLC verified purity of 99.60% for PA. A HPLC-MS/MS method was developed and validated for quantification of the LOX pathway products; LTB4, 5- and 12-HETE. An in vitro validation assay shows that quantitative determination of the analytes is linear in the range of 126–40000 pg/mL, and accuracy and precision met the acceptance criteria.

Conclusion: Quantification of LTB4 and HETEs in Capan-2 cell line, before stimulation with A23187 showed a weak signal for the analytes, indicating the presence of these analytes in the cell samples and the feasibility of the method. Further optimization of the extraction method for intact cellular lipids is needed, including improved methods for membrane rupture and modifications of the final sample preparation method to increase the yield prior to HPLC-MS/MS quantification. These optimizations are in progress and will then be applied to cells stimulated with A23187 with or without treatment with PA.

Chitinase-3-like-1 Protein Overexpression Enhances Breast Cancer Metastasis to the Lung

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Introduction: Breast cancer is the most common cancer in women. The prevalence of pulmonary metastasis is 3-fold greater in patients with chronic pulmonary inflammatory conditions. Chronic inflammation is one of the hallmarks of asthma, a disease of the airways. Currently one out of ten women suffers from asthma and will have a greater propensity to develop aggressive metastatic breast cancer. Published results indicated that one of the molecules that aggravate the inflammation in asthma is a glycoprotein known as Chitinase 3-like protein 1 (CHI3L1), a molecule that is associated with poor prognosis in metastatic breast cancer patients. We hypothesize that CHI3L1-induced pulmonary inflammation generates the proper environment for recruiting circulating breast cancer cells, thus increasing the rate of metastasis to the lung.

Material and Method: To determine if inflammation associated with CHI3L1 in the lung alters the pulmonary environment to accelerate metastatic growth, pulmonary inflammation is induced in BALB/c mice by ragweed allergen sensitization and injected with overexpressing CHI3L1 breast cancer cells. Results and Discussion: We show that there are elevated levels of CHI3L1 in circulation in increased tumor growth, higher levels of metastasis to the lung with shorter survival rates in mice with asthma. However, the direct role of CHI3L1 in promoting metastasis is not clearly delineated.

Conclusion: The studies presented here will provide a greater understanding of the role of CHI3L1 in enhancing metastasis and the tissue-specific molecular signals that act to exacerbate metastasis under conditions of chronic inflammation.
Neuroblastoma Cells Modulate Chemokine Receptor Repertoire of Resting NK Cells

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Introduction: Natural Killer (NK) cells represent important effectors against hematological and non-hematological malignancies. They are highly efficient in killing of tumor cells and are effective against tumor and stem cell properties, the ultimate target of both conventional and innovative therapies. Clinical evidences however, show that, in vivo, tumors could avoid NK-mediated attack. Indeed it has been shown that NK cell cytolytic activity can be downregulated by soluble mediators released by tumor cells either by immune cell types recruited at the tumor site. The observation that NK cells are often scarce within tumors, despite high local levels of chemokines prompted us to analyze whether tumor cells could avoid NK-mediated immunosurveillance by modulation of NK cell chemokine receptor repertoire. This study has been focused on immunomodulatory role of Neuroblastoma (NB), a poorly differentiated extracranial neuroectodermal tumor that accounts for 15% of all childhood cancer deaths.

Material and Methods: NK cells were purified from peripheral blood mononuclear cells of healthy donors by Human NK Cell Isolation kits. NK cell lines utilized and by the use of mAb-mediated blocking of specific chemokine receptors. Chemokin_analysis of neuroblastoma conditioned-NK cells was evaluated by standard assays.

Results and Discussion: Freshly purified human NK cells were cultured under trans-well conditions in the presence of NB cell lines. After co-cultures, NK cells were analyzed for the expression of a panel of chemokine receptors known to regulate their migratory capacity. This analysis showed that NB-conditioning up-regulates on NK cells CXCR3 and CXCR4 expression. On the contrary NB-conditioned NK cells were characterized by a significative decrease in CXCR1 expression as compared to unconditioned NK cells. Thanks to the analysis of the soluble factors released by the different NB cell lines utilized and by the use of mAb-mediated blocking of specific soluble factors we identified the mediator responsible for the described effect. Interestingly the described immunomodulatory feature was shared only by some of the several NB cell lines utilized.

Conclusion: Our study showed that neuroblastoma cell lines are capable of modulating the chemokine receptor repertoire of resting human NK cells. Since CXCR4 and CXCR1 represent crucial receptors driving bone marrow egress and bone marrow homing of NK cells, the described effect might anchor NK cells in the bone marrow, limiting their recruitment at tumor site. Thus our study might indicate a novel mechanism of tumor-evasion.

CD200 Expression and Fopx3+ Regulatory T Cells Level in Acute Myeloid Leukemia Patients Associated With Poor Prognosis

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Background: CD200 is an immunosuppressive molecule which over-expressed in some hematologic malignancies such as B-cell chronic lymphocytic leukemia (B- CLL) and multiple myeloma. It has been also shown to be an independent prognostic factor for patients with acute myeloid leukemia (AML). In this current study, simultaneous CD200 expression and fopx3+ regulatory T cells level were investigated in Iranian patients with AML.

Material and Methods: CD200 and its receptor (CD200R) expression levels were examined on bone marrow and peripheral blood leukemic cells obtained from 57 AML patients by Flow cytometry. This technique was used to determine the frequency of fopx3+ regulatory T cells in these patients. ELISA method was performed to investigate TGF-β and IL-10 production level in sera of patients.

Results: Correlation analysis demonstrated simultaneously increasing of fopx3+ regulatory T cells and CD200 expression in AML patients (p = 0.003; r = 0.73). Our clinically poor prognosis patients have shown both higher expression of CD200 and frequency of fopx3+ regulatory T cells (p = 0.01 and p = 0.03, respectively). Moreover, the serum concentration of TGF-β (but not IL-10) was significantly correlated with the expression of CD200 on leukemic cells (p = 0.02).

Conclusion: These data signify the CD200 roles in repression and suppression of anti tumor immune system response by stimulation of regulatory mechanisms in AML patients and can suggest that CD200 may have prognostic value in this malignancy.

Ovarian Malignant Ascites-derived Lymphocytes Stimulated With Prothymosin Alpha or Its Immunoactive Decapeptide Lyse Autologous Tumor Cells in Vitro and Retard Tumor Growth in SCID Mice

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Introduction: Tumor-associated lymphocytes (TALs) present in effusions of cancer patients exhibit impaired activities, due to the immunosuppressive environment of the ascites. Means to enhance their cytotoxicity against autologous tumor cells are of clinical importance. Prothymosin alpha (proTα), a 109 residue long acidic polypeptide with immunomodulatory activity, has been previously shown to synergize with cytokines (eg. IL-2, IFN-γ) and increase the cytotoxicity of cancer patient-derived PBMC, particularly the specific lysis of tumor cells by activated CD8+ T lymphocytes. ProTα’s immunoreactivity is exerted by the carboxy-terminal decapetide, proTα(100–109). Using TALs and autologous tumor cells from ovarian cancer patients, we studied the in vitro effect of proTα and proTα(100–109) in synergy with IL-2, in MLTCs set up for 7–21 days. Furthermore, the in vivo antitumor activity of MLTC-stimulated TALs, was tested in SCID mice inoculated with human ovarian tumor cells.

Material and Methods: TALs: Tumor cell-conditioned TALs (TALs) comprising of 20 patients with epithelial ovarian adenocarcinomas and co-cultured, at a TAL:tumor cell ratio of 10:1, in the presence of 200 ng/ml proTα or 25 ng/ml proTα(100–109) in synergy with IL-2 (100U/ml). As control, a scrambled decapetide with the same amino acids as proTα(100–109) was used. The cytotoxic activity of MLTC-derived TALs was determined by 51Cr release assays, using as targets autologous tumor cells, SKOV-3 and Daudi cells. TALs from selected patients (n = 3), in vitro stimulated for 6 days with proTα or proTα(100–109), were administered intraperitoneally (3 cycles at weekly intervals) in SCID mice inoculated with each patient’s autologous tumor cells.

Results: ProTα and its immunoactive peptide proTα(100–109), enhanced the cytotoxic activity of TALs against autologous tumor and SKOV3 cells in vitro, but marginally affected the lysis of the LAK-sensitive Daudi cells. No difference was recorded when the same TALs were stimulated with the scrambled peptide. The effect of proTα and proTα(100–109) was higher after 7–14 days of stimulation, whereas TAL cytotoxicity was significantly decreased after 21 days. Mice administered TALs, ex vivo activated with proTα or proTα(100–109) for 8 days, showed a relatively lower tumor invasion rate and a prolongation of their survival, compared to control mice. Our results suggest that proTα and proTα(100–109) can activate tumor-specific T lymphocytes in MLTCs set up with TALs from malignant effusions.

Conclusion: Our data demonstrate that proTα and proTα(100–109) can enhance the depressed cytotoxicity of TALs against autologous tumor cells in vitro and retard tumor growth in SCID mice. Thus, both, peptides, in the presence of tumor antigens, can stimulate anticancer-specific immune responses.

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Prothymosin Alpha and Its Carboxy-terminal Decapeptide ProTα(100–109) Induce Th1-type Tumor-specific Immune Responses

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Background: The biologic response modifier prothymosin α (proTα) enhances immune responses of lymphocytes by ligation toll-like receptor 4, expressed on innate immune cells. T cells and decoy proTα(100–109) comprises the immunomodative fragment of the polypeptide, being equally effective to the intact molecule in stimulating T cell functions. We have previously shown that human dendritic cells (DC) that mature in the presence of proTα or proTα(100–109) exhibit the characteristic mature-DC phenotype (eg. upregulation of CD83, CD83, CD86). Therefore, we tested the functionality of these DC, i.e., whether they can selectively expand tumor-antigen-specific CD4+ and CD8+ T cells, decreasing of naive lymphocytes populations of healthy donors. Tumor cells from 3 HLA-matched donors were differentiated to DC with GM-CSF and IL-4, matured with TNF-α (standard), proTα or proTα(100–109), loaded with the peptides and used to stimulate autologous T cells. After 3 rounds of stimulation with similarly matured and pulsed DC, HER-2/neu...
specific T cells were intracellularly stained for TNF-α, IFN-γ, IL-2, IL-4 and IL-17. They were also evaluated for their specific cytotoxicity and proliferation.

Results: An increased percentage of HER-2/neu-specific CD4+ and CD8+ T cells, expanded upon stimulation with proTNF-α or proTNF(100-109)-matured DC, produced IFN-γ, TNF-α and IL-2, at percentages equal to those induced by TNF-α-matured DC. On the contrary, the percentages of T cells that were stained positive for IL-4 and IL-17 were marginal. CD8+ T cells also exhibited enhanced lysis of target cells expressing HER-2/neu 980g and CD4+ T cells increased proliferation when challenged with stimulator cells expressing HER-2/neu 980T.

Conclusion: In the presence of tumor (HER-2/neu)-specific antigens, proTNF-α and proTNF(100-109)-matured DC are functionally competent, induce Th1-type immune responses and stimulate the expansion of naive T cells recognizing specifically the HER-2/neu antigen. The in vivo enhancement of cytotoxicity and proliferation of the antigen-specific CD8+ and CD4+ T cells via proTNF-α and proTNF(100-109), implies that the peptides may function similarly in preclinical animal models in vivo.

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1079 TFN-TNFR Interactions Influence Tumor Growth and Metastasis by Manipulating Regulatory T Cell Homeostasis

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Introduction: Tumor necrosis factor (TNF) signals via two receptors, TNFR1 and TNFR2, and was originally identified due to its anti-tumorigenic properties. Nevertheless, later on other studies showed that TNF can also drive tumor growth and metastasis.

Material and Methods: To assess the role of TNF-TNFR interactions in tumor growth and metastasis, we used two syngeneic in vivo bioluminescence imaging (BLI)-based mouse tumor models in conjunction with exogenous TNF treatment, TNF/TNFR knockout mice and bone marrow chimeras. Mice were injected i.v. with luciferase-expressing B16-F10 cells or orthotypically with luciferase-transfected Panc02 pancreatic cancer cells. The growth of B16-F10 lung metastases and primary pancreatic tumors was assessed using BLI. Finally, tumor-bearing organs were imaged ex vivo and analyzed by flow cytometry and immunofluorescence microscopy.

Results and Discussion: In the B16-F10 model, treatment of mice with exogenous TNF markedly enhanced tumor metastasis resulting in both elevated luciferase signals in vivo and increased lung metastases numbers. Metastasis correlated with an induction of regulatory T cells (Tregs) in the lungs and experimental depletion of Tregs attenuated B16 metastasis. In comparison to wild-type mice, TNFR1 ko mice showed elevated metastasis and increased numbers of Tregs. Loss of either TNF or TNFR2 on immune cells reduced both metastasis and the number of Tregs. In the pancreatic tumor model, loss of TNFR1 strongly promoted tumor growth and increased the infiltration of Tregs into the tumor tissue, with the majority of these cells being TNFR2-positive. Tumors in TNFR1 ko mice also showed an increase in vascular density compared to the other genotypes. Influx of CD8+ T-cells into the tumors inversely correlated with the numbers of Tregs. We suggest that TNF activates TNFR2 on regulatory T-cells and thereby contributes to tumor-associated immune suppression. Furthermore, the enhanced metastasis and tumor growth observed in TNFR1 ko mice perhaps reflect reduced activity of other anti-tumoral acting cell types that are stimulated via TNFR1.

Conclusion: TNF is involved in the homeostasis of Tregs, and by inducing these cells, TNF creates a more tolerogenic environment and suppresses tumor immunosurveillance. The induction of suppressive Tregs could be seen as a feedback-loop in which TNF down-regulates its own pro-inflammatory functions.

1080 Dacarbazine-mediated Upregulation of NKG2D Ligands on Tumor Cells Activates NK and CD8 T Cells and Prevents Melanoma Growth

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Background: Dacarbazine (DTIC) is a cytotoxic drug widely used for melanoma treatment. However, the putative contribution of anticancer immune responses in the efficacy of DTIC has not been evaluated.

Material and Methods: We tested the immune dependency of DTIC antitumoral efficiency in an experimental mouse model of melanoma as well as the modifications of immune actors after DTIC treatment.

Results: We unexpectedly found that both NK and CD8+ T cells were indispensable for DTIC therapeutic effect. While DTIC did not directly affect immune cells, it triggered the upregulation of NKG2D ligands on tumor cells, leading to NK-cell activation and IFNγ secretion in mice and humans. NK-cell derived IFNγ subsequently favored upregulation of MHC class I molecules on tumor cells, rendering them sensitive to cytotoxic CD8+ T cells. Accordingly, DTIC markedly enhanced CTLA4 inhibition efficacy in vivo in an NK-dependant manner.

Conclusions: These results underscore the immunogenic properties of DTIC and provide a rationale to combine DTIC with immunotherapeutic agents that reave immunosuppression in vivo.

1081 SDF-1 Expression is Associated With Tumor Invasiveness in Recurrent Glioma After Radiotherapy

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Background: Radiotherapy (RT) has been a critical and important element of treatment for high-grade glioma, but the recurrence remains the main obstacle for clinical successes. This study used a murine glioma model to investigate the roles of stromal-derived factor-1 (SDF-1/CXCL12) on tumor invasiveness in recurrent glioma after RT.

Material and Methods: Two micro-illers PBS containing 1×105 cells of a murine high-grade astrocyoma cell line, ALTSIC1, were inoculated intrathecally into 6- to 8-week-old C57BL/6 mice. The tumor histology, microvascular density (MVD), number and phenotypes of tumor-associated macrophages (TAMs), and the expression of SDF-1 were examined by immunohistochemical (IHC) stain and fluorescence-activated cell sorter (FACS).

Results: Local brain irradiation prolonged the survival day of ALTSIC1 tumor-bearing mice, but mice died mainly from increased invasive tumors. IHC study revealed that those invasive borders and islands after RT have increased MVD, TAM number, and SDF-1 expression in both tumor cells and TAMs than those in control ALTSIC1 tumors. When SDF-1 expression in ALTSIC1 tumors was suppressed by specific siRNA, mice bearing these tumors had longer survival day after RT than parental tumors, and remarkable decreases of invasive tumors in these tumors were observed.

Conclusions: This study indicates that tumor-secreted SDF-1 plays a critical role on tumor invasiveness in high-grade glioma after RT, and its effects are possibly through on TAM mobilization and tumor revascularization. This study is supported by NHRI-EX101-10132B.

1082 Periforin-dependent Immunossurveillance of ErbB2+ Carcinomas in Female and Male Mice

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Background: Tumor immunossurveillance rests on T cell and NK cells in both mice. In the case of ErbB2+. After irradiation, periforin and perforin secretion is markedly enhanced control expression of these genes.

Methods: While BALB-neuT male mice rarely develop mammary carcinomas, all BALB-neuT female mice develop, multiple, fast-growing, invasive carcinomas in all mammary glands. When we back-crossed BALB-neuT mice with pfpKO BALB/cic mice, the tumor onset in male and female BALB-neuT and BALB-neuTpfpKO mice was evaluated.

Results: An accelerated onset of mammary carcinomas and a higher number of carcinomas per mouse were found in BALB-neuT/pfpKO female mice. Moreover, while BALB-neuT male mice rarely develop mammary carcinomas, surprisingly, the great majority of BALB-neuT-pfpKO male mice develop them. BALB-neuT/pfpKO male mice display impaired re-adoption of mammary gland ductules in the last period of fetal life and in the newborn mice. Mammary cancer develops from rudimental ductules expressing the ErbB-2 oncogene and persisting in adult BALB-neuT/pfpKO male mice.

Conclusion: Present data show that pfp-mediated mechanisms play crucial role in mammary gland morphogenesis and in the immunossurveillance of ErbB-2+ carcinomas. Altered re-adoption of mammary ductules may allow ErbB2+ cells to persist in adult male mice and give rise to carcinomas.
**DPPIV – Serum Activity and Expression on Lymphocytes in Different Hematological Malignancies**

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**Introduction:** Dipeptidyl peptide IV is implicated in regulation of initial steps of carcinogenesis, in invasion and metastasis of cancer. Changes in DPPIV activity and/or expression have been associated with various malignant diseases. The aim of this research was to determine the activity of DPPIV in serum, as well as the percentages of CD26+ lymphocytes and CD26+ overall white blood cells in patients with different hematological malignancies and in healthy control subjects.

**Materials and Methods:** This study involved 51 patients with non-Hodgkin lymphoma (NHL), 35 patients with leukemia, 18 patients with plasmacytoma and 22 patients with myeloma multiplex (all before therapy). The control group consisted of 40 healthy persons. Determination of DPPIV serum activity was performed by direct photometric method, while CD26 expression was analyzed using flow cytometry.

**Results and Discussion:** There is a statistically significant decrease in serum DPPIV activity, percentages of CD26+ lymphocytes, percentages of CD26+ overall white blood cells and in the lymphocytes percentages in patients with NHL in comparison to healthy control subjects (p < 0.0000003, p < 0.008, p < 0.0000001, p < 0.0000006, respectively). Furthermore, patients with leukemia had statistically significant lower activity of DPPIV, in serum (p < 0.0068) and significant decrease in the percentages of CD26+ lymphocytes (p < 0.0000005) in relation to healthy controls. It should be stressed that statistically significant decrease in serum DPPIV activity was observed in the groups of patients with plasmacytoma and myeloma multiplex (p < 0.002, p < 0.0009) compared to healthy control group. Additionally, patients with plasmacytoma had significantly lower percentages of CD26+ lymphocytes and overall immunocompetent cells (p < 0.003, p < 0.007), while patients with myeloma had decreased percentages of CD26+ overall white blood cells (p < 0.005) and decreased percentages of lymphocytes (p < 0.05).

**Conclusion:** Data from our study show statistically significant decrease in serum DPPIV activity in the groups of patients with NHL, leukemia, plasmacytoma and myeloma multiplex. Lower activity of DPPIV was associated with decreased expression of DPPIV (as CD26) on lymphocytes in patients with NHL, leukemia and plasmacytoma. Further research is needed in order to evaluate the significance of decrease in serum DPPIV activity and CD26 expression on lymphocytes in development and progression of investigated hematological malignancies.

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**Necadherin and LMP-1: A Shared Immunotherapy to B-cell Lymphomas**


**Introduction:** A phase II study was performed to assess the role of patients’ immune profile in the activity of a trastuzumab (T)-based, non anthracycline hormonal therapy and chemotherapy. In case of progression, T was discontinued and anthracyclines were planned. Blood samples were collected at diagnosis and every 3 months for 1 year then yearly. The % of NK cells, T cells and Treg cells was evaluated by flow cytometry, and serum levels of cytokines and chemokines were assessed by ELISA and ELispot technology. Circulating CD8 T cells specific for a broad spectrum of tumor-associated antigens (Her2, muc-1, mammaglobin-A, trag-3, survivin, bcl-xl) were determined using ELispot and in vitro T-mediated autologous body-cell dependent cytotoxicity (ADCC) was assessed using patients’ PBMCs.

**Results:** Globally, 34 pts were enrolled (median age 46 yrs). The rate of overall clinical responses was 91%, with 53% of pathological complete responses (pCR) and 38% of partial clinical responses (pPR) with an immune profile similar to that of healthy women, whereas higher numbers of Treg cells (p < 0.02), NK cells (p < 0.03), lower T cell numbers (p < 0.01) and lower amounts of serum IL-2, IL-6, and IL-8 were found in a control group of untreated HER2+ pts. Moreover, higher numbers of spontaneous CD8+ T cells specific for 13 HLA-A*0201 epitopes were observed in the whole series if compared to HER2- cases. Notably, pCR and >CR pts showed higher numbers of epitope-specific CD8+ T cells throughout the NC treatment. Moreover, pts achieving pCR showed at diagnosis a significantly higher efficiency of T-mediated ADCC compared to pts with pPR (p < 0.05). The treatment also induced a progressive increase in the number of NK cells and in the efficiency of T-mediated ADCC.

**Conclusions:** NC with P and T induces high rates of pCR with no cardiovascular toxicity. This clinical benefit is favoured by the higher NK cell and the increased efficacy of T-mediated ADCC in preserving event-free survival and the involvement of P in inducing NK cells’ NF-κB nuclear translocation are under investigation.

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**Generation of EBV-specific CTL Lines Enriched in BARF1 Specificities for Improved Adoptive Immunotherapy of Nasopharyngeal Carcinoma**


**Introduction:** The extent of objective clinical responses induced by EBV-specific adoptive immunotherapy in patients with nasopharyngeal carcinoma (NPC) is still unsatisfactory due to the lack of sufficiently high efficacious antigens that can be targeted and their low immunogeneity. We demonstrated the presence of strong CD4+ and CD8+ T cell responses against the EBV-encoded BARF1 protein, highly expressed in NPC patients’ tumor cells. Moreover, the identification and validation of several BARF1-specific CTL epitopes provide the rationale to exploit this protein as tumor-associated antigen to improve the efficacy of current EBV-specific immunotherapy protocols.

**Material and Methods:** Several approaches were used to assess whether appropriate manipulation of autologous LCLs may render these cells able to enrich conventional EBV-CTL lines in BARF1-specific effectors, without depleting specificities to other relevant latent EBV antigens. In particular, we used as antigen presenting cells LCLs treated with either TPA and butyric acid (TPA), doxorubicin (DX) or cisplatin (CPL), in order to induce abortive EBV lytic cycle. Un-treated and BARF1-enriched LCLs were used to generate polyclonal CTL cultures and the expression of relevant EBV genes was monitored by RT-PCR before each re-stimulation.

**Results:** LCLs treated with DX displayed a 2.7 fold increase BARF1 mRNA expression, while retaining moderate levels of LMP1 (2.1 fold increase) and other latent genes. Moreover, while CTLs generated with un-treated-LCLs showed low levels of BARF1-specific lysis (≤5%), CTL lines obtained with treated LCLs induced significant BARF1-specific cytotoxic activity against both HLA-A*0201-transduced c866-1 NPC cells lines (endogenously expressing BARF1) and T2-A2 cells loaded with BARF1-derived peptides. Notably, CTL cultures generated with DX-treated LCLs induced 40- to 60%-specific lysis against BARF1-loaded targets and 30% of lysis against c866-1/A0201 cell line (cPL-CTLs displayed 16% and 19% lysis respectively). More importantly, despite the up-regulation of several EBV latent genes, DX-LCLs did not affect the generation of CTLs specific for different LMP-1 or EBNA1 epitopes. We have also shown that BARF1-specific CTL lines can be easily obtained from NPC patients with different HLA backgrounds.

**Conclusions:** These findings further confirm the relative immunodominance of BARF1 CTL epitopes and support the immunogenic properties of DX, providing the rationale for the use of EBV-CTL lines enriched in BARF1 specificities for the treatment of resistant or relapsing NPC.

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**IGHV1-69 as a Promising Candidate for the Development of a Shared Immunotherapy to B-cell Lymphomas**

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**Introduction:** B-cell Non-Hodgkin Lymphomas (B-NHL) are a heterogeneous group of cancers, broadly diffuse worldwide and often relapse after standard treatment and rituximab. Therapeutic vaccines targeting B-NHL idiotype (Id) represent a promising approach, limited by being patient-specific and by the lack of eligible and cost-effective antigens markers. Nevertheless, the molecular characterization of different lymphoid tumor histotypes revealed a set of stereotyped immunoglobulins among distinct B-cell lymphoma types. On this ground, we focused our attention on the IGHV1-69 protein, frequently expressed in NPC diagnosis, pts achieving pCR and in chronic lymphocytic leukaemia and auto-immunity related lymphoproliferations.

**Materials and Methods:** Within IGHV1-69 sequence we identified 13 potential CTL (cytotoxic T lymphocyte) epitopes and synthesized the
corresponding pentamers (Pent). We assess by flow cytometry the presence of epitope-specific T-cell responses in peripheral blood of patients with hGHV1−69+ familial lymphoproliferative diseases and validated these data in IFN-γ ELISPOT (Enzyme-linked immunosorbent spot). Finally, we generated CTL lines for each epitope, stimulating donors and patients PBLs with peptide-pulsed cell lines and testing their specificity in cytoytic assays.

**Results and Discussion:** Interestingly, the hGHV1−69 Pent+ population in patients was generally larger than in donors, supporting the existence of spontaneous memory T-cell responses against hGHV1−69. Surprisingly, in some patients we observed higher numbers of hGHV1−69+ Pent− cells than control virus-Pent+ cells, which also displayed higher functionality in ELISPOT assays compared to viral-specific T cells. Moreover, peptide-specific CTL lines showed a weak but specific lysis against peptide-pulsed targets, especially when derived from patients’ PBLs; in addition, we observed specific lysis also towards an hGHV1−69 naturally expressing cell line, suggesting that hGHV1−69 memory T cell responses could be boosted for therapeutic purposes.

**Conclusion:** These results show that hGHV1−69 constitutes a potential target for the development of a subset-specific Id vaccine. Furthermore, Pent and ELISPOT immune-monitoring may partially overcome the main limitations of current Id-targeting vaccinations and further improve their clinical efficacy.

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**1085 In-depth Analysis of Cancer HLA-I Peptidomes**

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**Introduction:** The Human Leukocytes Antigen class I (HLA-I) molecules are transporters that carry peptides from the cytoplasm to the cell’s surface for surveillance by circulating T lymphocytes. In healthy cells, the HLA-I peptides originate from normal proteins, and the immune system ignores them. However, infected cells or cancer cells, peptides originating from viral or cancer-related proteins can elicit an immune response. Cancer-related peptides have been extensively studied because of their potential role as immunotherapeutic cancer vaccines. Furthermore, apart from being bound to the cell surface, HLA-I molecules also occur in the plasma as soluble complexes with their bound peptides and these peptides can be used as biomarkers for cancer. In this study large scale HLA-I peptidomes are identified from cancer cells, by state of the art mass spectrometry instruments and methodologies, for the identification of new cancer related HLA-I peptides. Such peptides can be further evaluated as biomarkers in a targeted analysis in soluble HLA peptidomes from plasma samples of patients.

**Material and Methods:** HLA-I complexes are mass-affinity purified from several cancer cell lines, and the enriched HLA peptidomes are analyzed by reversed phase chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a quadrupole Orbitrap mass spectrometer (Q Exactive). Peptide fragments are analyzed at high resolution and mass accuracy in the Orbitrap analyzer. The peptides are identified using the MaxQuant software, and cancer related peptides are selected based on databases and literature search.

**Results:** The Higher energy Collisional Dissociation (HCD) fragmentation method used in the Q Exactive results in MS/MS spectra covering the complete mass range and fragments that are measured with nearly hundred-fold increased mass accuracy compared to low resolution ion trap measurements. This significantly improves the quality of peptide identification and results in significantly higher confidence in HLA-I identified peptides, among them potential new cancer related peptides. The obtained dataset is the basis of an in-depth correlation study between the HLA-I peptidome and the cellular proteome of cancer cells, which will improve our understanding of the cellular proteins of origin of these peptides.

**Conclusions:** In depth mass spectrometry analysis of HLA-I peptides by the HCD fragmentation method used in the Q Exactive significantly improves the quality of peptide identification. This methodology facilitates the measurement of cancer related peptides for further targeted analysis in tumors or plasma samples, for their evaluation as potential immunotherapeutics and as biomarkers.

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**1089 Intraocular Microscopy Identifies Antibody-dependent Phagocytosis by Macrophages as Main Effector Mechanism of Tumor Necrosis in Familial Liver Lesions**

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**Introduction:** Development of surgery-induced liver metastases from colorectal tumors is a complication in patients. Surgery was paradoxically shown to enhance adhesion of circulating tumor cells in the liver. Thus, minimal residual disease is present in patients post-surgically, which renders them exceptionally suitable for adjuvant immunotherapy. In a previous study, we observed in the livers of mice that ~80% of tumor cells were in contact with liver macrophages, Kupffer cells (KCs). Furthermore, in the livers of mice, which were treated with a tumor-specific monoclonal antibody (mAb), the contact between KCs and tumor cells was increased to approximately 90%. Surprisingly, this modest difference completely prevented liver metastases development.

**Material and Methods:** To study the exact mechanisms of mAb therapy, we used real-time intraocular microscopy of the liver of mice and in vitro live cell imaging and Imagestream technology to visualize the mode of action in more detail.

**Results and Discussion:** KCs nibbled at tumor cells in untreated mice and were only able to take up small parts of tumor cells. They were however unable to prevent tumor growth. By contrast, KCs phagocytosed complete tumor cells within 60 minutes when mice were treated with mAb. Three-dimensional reconstruction of liver sections demonstrated that tumor cells had been degraded into small particles, whereas large tumor cell clusters were observed in untreated mice. Moreover, therapeutic efficacy was abrogated in KC-depleted mice.

**Conclusion:** Thus, mAb therapy-dependent phagocytosis by KCs was the main mechanism involved in therapeutic efficacy, as antibody treatment leads to improved tumor cell recognition and uptake, which prevented the development of liver metastases. As such, peri-operative antibody treatment of patients undergoing surgery for colorectal cancer may significantly improve patient outcome.

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**1090 Tumor-associated Antigen Validation in Uterine Tumors**

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**Background:** Tumor-associated antigens (TAA) are crucial players in immunotherapeutic strategies. Quite some TAA have been described in different cancer types, some in uterine tumors. Although having been applied for other cancers, little research has been done to evaluate TAA in an immunotherapeutic setting in uterine tumors. This research focuses on the validation of TAA for their applicability in a dendritic cell-based vaccine for uterine cancer.

**Material and Methods:** 6 different TAA were analyzed in uterine tumors: BORIS, MUC1, survivin, hTERT, sp17 and MAGE-A3. TAA expression was analyzed by qRT-PCR using snap-frozen tissue and IHC using paraffin-embedded tissue. Samples varying in tumor grade or stage as well as different histological subtypes were included. Statistical analyses were performed using the non-parametric Kruskal–Wallis test.

**Results and Discussion:** We show that for endometrial carcinoma (EMC) 40-100% of cases express BORIS mRNA, 90% MAGE-A3, and 100% of cases of primary and metastatic EMC (p < 0.01) as well as metastatic lesions (p < 0.01). Survival analysis shows an upregulation in recurrent EMC compared to primary EMC, suggesting a growth advantage of the tumor cells causing the recurrence. Remarkably, hTERT is upregulated in primary (p < 0.05) and recurrent EMC (p < 0.01) as well as metastatic lesions (p < 0.01). Survival analysis shows an upregulation in recurrent EMC compared to primary EMC, suggesting a growth advantage of the tumor cells causing the recurrence. Therefore, endogenous T and B cell responses against the antigens are currently under evaluation as well as further validation of these TAA in a panel of normal tissues.
Intracellular Expression of Interleukin-10 (IL-10) in Patients With Ovarian Cancer

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Introduction: The identification and characterization of cellular target structures are prerequisites for the development of novel immunotherapeutic strategies. Like adoptive T cell transfer, vaccines, engineered antibodies or small molecule compounds for cancer treatment, suitable targets represent the so-called tumor-associated antigens (TAA). The role of intracellular expression of IL-10 in ovarian cancer patients and cancer tissues suggests that these are cells that might be considered as cancer targets.

Material and Methods: CD4+ and CD8+ T cells producing IL-10 in ovarian cancer patients (OVC). The percentage of CD4+ T cells producing IL-10 was significantly higher in the tumor tissue (1.91%) than in the peripheral blood (0.24%). Additionally, it was shown that the percentage of CD4+IL-10+ T cells was significantly higher in the peripheral blood (0.4%) compared with the peripheral blood (0.08%). There were no significant differences between the percentage of CD6+IL-10+ T cells between the peripheral blood (0.8%) and the peripheral blood (0.24%) and cancer tissue (0.4%).

Results: We demonstrated the presence of IL-10+ T cells in both CD4+ and CD8+ T lymphocytes in ovarian cancer patients. The highest percentage of CD4+IL-10+ T cells was observed in the cancer tissue, while two other genes, LUM and SFRP2, showed a 14- or 19-fold over-expression, respectively. Correlation with clinical data to determine the prognostic relevance of humoral immune responses and/or gene expression levels is currently being done.

Conclusion: The serological multiplex screening of more than 200 patients’ sera showed that IL-10 is also secreted by different cancer types including ovarian cancer. In patients with advanced ovarian cancer IL-10 is overexpressed in sera and ascites. The role of intracellular expression of IL-10 in ovarian cancer microenvironment remains unclear. The aim of study was to compare the percentage of peripheral blood (PB), peritoneal fluid (PF) and cancer tissue CD4+ and CD8+ T cells producing IL-10 in ovarian cancer patients. Peripheral blood, peritoneal fluid and cancer tissue mononuclear cells were obtained by centrifugation and digested with an enzyme mixture. CD4+ and CD8+ T cells producing IL-10 detection was performed by using the Kaplan–Meier test and multivariate analysis by using Cox regression analysis.

Background: Interleukin-10 (IL-10) is an immunosuppressive cytokine produced by a variety of cells such as monocytes, macrophages, B- and T-lymphocytes. It was proved that IL-10 is also secreted by different cancer types including ovarian cancer.

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Conclusion: Our studies identified four candidate proteins from a pool that either induced antibody responses in patients with pancreatic cancer or are overexpressed in pancreatic cancer tissue. Statistical analysis will reveal the prognostic potential of the four candidates.

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Results and Discussion: The percentage of peripheral blood (PB), peritoneal fluid (PF) and cancer tissue CD4+ and CD8+ T cells producing IL-10 in ovarian cancer patients (OVC) was significantly higher in the peripheral blood (0.24%). Additionally, it was shown that the percentage of CD4+IL-10+ T cells was significantly higher in the peripheral blood (0.94%) compared with the peripheral blood (0.08%). There were no significant differences between the percentage of CD6+IL-10+ T cells between the peripheral blood (0.08%) and the peripheral blood (0.24%) and cancer tissue (0.4%).

Results: We demonstrated the presence of IL-10+ T cells in both CD4+ and CD8+ T lymphocytes in ovarian cancer patients. The highest percentage of CD4+IL-10+ T cells was observed in the cancer tissue, while two other genes, LUM and SFRP2, showed a 14- or 19-fold over-expression, respectively. Correlation with clinical data to determine the prognostic relevance of humoral immune responses and/or gene expression levels is currently being done.

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Additionally, liver vessel integrity was interrupted, as expression of the tight junction molecules ZO-1 and Claudin-5 were decreased after laparotomy and subfascial colectomy, indicating loss of cell-cell contact after abdominal surgery. This was likely due to increased macrophage activity, because we previously demonstrated that activation of macrophages by LPS led to disrupted liver vasculature.

In conclusion, our results support that macrophages were activated by a systemic factor in the plasma of operated rats, which led to impaired liver vasculature and augmented tumor cells adhesion and outgrowth. Exposure to tumor microenvironment may contribute to this process, as this will strongly activate macrophages. Understanding the precise mechanisms may aid the rational design of novel strategies to prevent liver metastases development after bacterial contamination, hereby improving prognosis of patients undergoing CRC resection.

**1005** The Evaluation of Natural Killer Cells in Ovarian Cancer Patients
A. Nowicka1, E. Rogala 1, I. Wertel 1, W. Piekarzcyk 1, J. Kotarski 1, S264 European journal of cancer 48, suppl. 5 (2012) S25–S288 Sunday 8

**Patients and Methods:** Flow cytometry was used to evaluate the percentage of Natural Killer cells from the peripheral blood (n = 34), peritoneal fluid (PF) and ovarian cancer tissue.

**Results:** The percentage of NK cells in the peripheral blood (PB), peritoneal fluid (PF) and ovarian cancer tissue was 11.09% ± 2.38%, 7.84% ± 0.97% and 17.67% ± 3.46%, respectively.

**Conclusions:** The lower percentages of NK cells in the peripheral blood of ovarian cancer patients may suggest that these cells may poorly migrate to the tumor microenvironment. The lower percentages of NK cells in the peritoneal fluid and cancer tissue may imply that tumor may have an influence on local immune response.

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**1006** Study on Oxidative Imbalance Associated Clinical Complications Among Breast Cancer Patients
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Breast cancer is one of the most common cancers in women of developed and developing countries. Oxidative stress arises when there is an imbalance between reactive oxygen species (ROS) and scavenging capacity of antioxidants, and it can induce and progress many diseases such as breast cancer. Oxidative stress, especially lipid peroxidation is known to be involved in carcinogenesis. Increased levels of lipid peroxidation products play a role in the early phases of tumor growth. LDL-cholesterol is more susceptible to oxidation in various pathologic conditions, which result in higher LPO (lipid peroxidation) and oxidative stress. HDL-cholesterol, on the other hand, is able to counterbalance the oxidative damage of LDL-cholesterol on cell membrane and prevent LPO. It has been suggested that HDL-cholesterol prevents both enzymatic and non enzymatic generation of O2-, HO2 and OH and thus acts as an anti carcinogenic and a powerful antioxidant. Present study attempts to associate Oxidative stress with lipid peroxidation among breast cancer patients.

**Methodology:** The study involves forty breast cancer patients who are attending Department of Radiation Oncology, Government Rajaji Hospital Madurai for the present study. The samples were collected at the informed consent of the patients. Serum samples were collected SGGT and Lipid profile was determined. Reactive Oxygen Species (ROS) was quantified using Nitro Blue Tetrazolium (NBT) reduction assay.

**Results:** Study involves distribution and statistical analysis confirms the existence of the extreme significance of oxidative stress associated lipid peroxidation among the study population (p < 0.0001) one way variance analysis (ANOVA). Study document existence of Correlation between increased oxidative stresses with Level of SGGT.

**Conclusion:** The excess productions of ROS establish the existence of liver dysfunction and higher risk of lipid peroxidation which complicate the process of carcinogenesis. Increased levels of lipid peroxidation among breast cancer patients prevents oxidative stress associated complication and the establishment of the net antioxidant status thereby minimizing the risk of Lipid peroxidation associated complication among breast cancer patients.

**1007** Role of PD-L1 in In-vitro Interaction Between T-cells and Ovarian Tumour Cells
J. Chatterjee1, N. Haslinda Abdul Aziz2, C. Maine1, C. Hayford1, L. Whilding1, A.J.T. George2, S. Ghaem-Maghami1, A. Imperial College Hammersmith Hospital Campus, Cancer and Surgery, London, United Kingdom, 2Imperial College Hammersmith Hospital Campus, Immunology, London, United Kingdom

**Introduction:** Ovarian cancer is the second most common and the most lethal malignancy of the female reproductive tract. Negative regulatory mechanisms within the tumour microenvironment inhibit tumour T-cell function, leading to evasion from any immune response against the tumour. PD-L1 is a cell surface immunoglobulin of the B7 super family. The binding of PD-1, expressed on tumour cells to PD-1 on activated T cells, is thought to result in an immunoinhibitory mechanism in this immune evasion process; though cytokines in the tumour microenvironment may also play a role. We have found high expression of PD-L1 at m-RNA and protein level in all severe ovarian cancer cell lines. Here we have investigated the role of PD-1/PD-L1 engagement in in-vitro CD3/CD28 stimulatory responses, using ovarian cancer cell lines.

**Material and Methods:** Tumour cells and supernatant from benign and malignant ovarian tumour cell lines were cultured with healthy CD3/CD28 bead stimulated T cells, with and without the presence of a blocking anti-PD-L1, or anti-PD-1 antibody and antibodies against key cytokines. This was repeated in a transwell model. T-cell proliferation was measured by flow cytometry looking at CFSE expression on CD3 cells on day 5.

**Results and Discussion:** We have shown that ovarian cancer cell lines that express PD-L1 do not appear to up regulate PD-1 on resting T cells. We found suppression of T-cell proliferation when co-cultured with ovarian cancer cell lines and partial reversal of T-cell suppression by blocking PD-L1 but not PD-1. This suppression was not observed when benign ovarian surface epithelial cells were used. The reversal was not increased by simultaneously blocking IL10, a key immunosuppressive cytokine in the tumour microenvironment. In order to understand whether this suppression of activated T-cell by malignant tumour cells is due to cell to cell contact or due to soluble factors, we performed the same co-culture experiment using a transwell model. This showed continued suppression of T-cell proliferation but no meaningful reversal was found when blocking PD-1 on T-cells or PD-L1 on T-cells.

We have also seen significant suppression of T-cell proliferation when using supernatant from malignant ovarian cancer cell lines. Preliminary data suggest that apoptosis may be induced in this model.

**Conclusion:** We have shown suppression of T-cell proliferation in activated T-cells co-cultured with malignant ovarian tumour cells. However, this suppression was only partially reversed with PD-L1 blockade suggesting a limited role of the PD-1/PD-L1 blockade in this experimental model. We have also shown supernatant from ovarian cancer cell lines suppress T-cell proliferation by soluble factors without direct cell to cell contact.

**1008** Adenosine Metabolism in T Cells in Melanoma and Pancreatic Cancer and Its Therapeutic Modulation
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**Background:** Extracellular ATP and adenosine (ADO) are well known signaling molecules that modulate the levels of intracellular calcium and cAMP. ATP can be converted into adenosine by ectonucleotidases CD39 and CD73 in a stepwise manner. Extracellular ADO activates A2A receptors, which leads to elevation of cAMP and suppression of effector T cell function. Recently, CD39 and CD73 expression and their activity have been linked to suppressive properties of regulatory T cells. This suggests that therapeutic inhibition of adenosine production and signaling could be a promising strategy to boost antitumor immune response.

**Methods:** Ret-transgenic mice were used as a model of melanoma. In the orthotopic model of pancreatic ductal adenocarcinoma (PDAC), highly tumorigenic Panc02 cells were injected directly into the pancreas
of immunocompetent mice. Analysis of CD73 and CD39 expression was performed by flow cytometry using FACS Canto II with FACS Diva Software (BD Biosciences). For the survival analysis REF Tumor-bearing mice were injected intraperitoneally with ACPG (CD73 inhibitor) at 20 mg/kg or with SCH58261 (A2a receptor antagonist) at 5 mg/kg three times a week for 60 days and monitored daily for tumor progression. Control group of mice with tumors of similar size received PBS.

**Results:** In the current study, we investigated the role of ectonucleotidases in various T cell subpopulations in mouse models of melanoma and PDAC. We confirmed that CD39 and CD73 are high expressed by regulatory T cells. We also detected ectonucleotidase expression on FoxP3+ conventional T cells (Tcon) and CD6 T cells. Importantly, CD39 and CD73 expression was strongly elevated on CD25+ Tcon and on all Tcon and CD8 T cells at the tumor site in both models. We hypothesized that inhibition of adenosine production or signaling should restore antitumor T cell response and thus lead to a positive clinical effect. To this end, we treated REF-Transgene tumor bearing mice with uA-methyleneadenosine 5’ diphosphate (APCP) or SCH58261. Indeed, this therapy resulted in a significantly prolonged survival of tumor bearing animals.

**Conclusions:** Our findings suggest that adenosine production by T cells is strongly increased in the tumor microenvironment. Targeting adenosine metabolism holds promise as a novel strategy for cancer immunotherapy. We hypothesize that adenosine production or signaling should restore antitumor T cell response and thus lead to a positive clinical effect. To this end, we treated REF-Transgene tumor bearing mice with uA-methyleneadenosine 5’ diphosphate (APCP) or SCH58261. Indeed, this therapy resulted in a significantly prolonged survival of tumor bearing animals.

**[1099] Induction of Anti-tumor Immune Responses by Ablation of the Primary Tumor With Pulsed Electric Currents**

I. Hochman1, H. Confino1, M. Efrati1, R. Korenstein2, H. Bernard2, R. Goldschmidt2, B. Klein2, P. Beckhove1.1 Tel Aviv University, Clinical Microbiology and Immunology, Tel Aviv, Israel, 2 Tel Aviv University, Physiology and Pharmacology, Tel Aviv, Israel.

**Background:** Ablation is a local treatment, which is used for in situ destruction of solid tumors. In situ tumor ablation can result in propagation of antigenic molecules, which can activate immunological reactions against the tumor that may subsequently destroy residual malignant cells. Thus, in order to both destroy the tumor and prevent metastases we investigate the immunotherapeutic approach which claims that the tumor can serve as its own antigenic vaccine provided that the tumors are effectively killed inside the body.

In our previous studies we performed electrochemical ablation of tumors using a treatment in which a train of unipolar-pulsed electric currents was delivered (pulsed electric current tumor ablation − PECTA).

**In this study we further defined the electrical parameters required for maximal ablation of experimental mammary adenocarcinoma and colon carcinoma by PECTA, and the manifestation of anti-tumor immunity following PECTA.**

**Materials and Methods:** Balb/c mice bearing subcutaneous tumors (7–9 mm in diameter) of murine mammary adenocarcinoma (DA3) or colon carcinoma (CT26) cells, were treated with 25–100 coulombs (C) per cm² of tumor tissue. Intra-tumoral electrodes delivering electric currents of 30 mA were used. PECTA mediated elimination of the primary tumors was measured and compared to surgical removal of the tumors. To test anti-tumor responses, mice cured by PECTA or surgery were reinfected with tumor cells (challenge assay), or splenocytes from such treated mice were mixed with tumor cells and implanted subcutaneously in naive mice (Winn assay).

**Results and Discussion:** Primary tumor ablation: A direct correlation was found between the amount of charge delivered and the rate of primary tumor ablation, and treatment with 100 C/ cm² cured 100% of the mice (ablation defined as no recurrence for 3–4 months).

Anti tumor immunity: DA3 bearing mice cured by PECTA cured mice were more resistant to the growth of a tumor cell challenge than surgery cured mice. Tumor volumes 12 days after the challenge were 77 mm³ in PECTA, and 125 mm³ in surgery treated mice.

Splenocytes from PECTA cured mice were more efficient in inhibiting tumor growth compared to splenocytes from surgery cured mice (Winn assay). Forty days after the injection of splenocytes/tumor cell mixture, 50% of the PECTA treated mice were tumor free, while all the mice treated by surgery developed tumors. The average tumor volume was 148 mm³ and 991 mm³ for PECTA and surgery treatment, respectively.

**Conclusions:** The results indicate that PECTA successfully ablated DA3 and CT26 tumors and stimulated the immune response against the tumor and metastatic lesions. PECTA together with chemotherapy and/or immuno-stimulatory agents can serve as treatment protocol given instead or in combination with surgery.

**[1101] Human Tumor Cells Inhibit T Cell Responses Through Expression of Carinoembryonic-antigen-related Cell Adhesion Molecule-6 (CEACAM-6)**

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**Introduction:** Although functionally competent and antigen-reactive CD8+ T-cells are evident in tumour patients, they hardly detect and deter tumour growth, indicating an immune-suppressive crosstalk between the tumour and the immune cells. Elucidation and subsequent targeting of such tumour-mediated immune evasion pathways is crucial for the clinical success of cancer immunotherapy. We here show that myeloma cells, as well as breast cancer cell lines, express cancer antigen-presenting molecule-6 (CEACAM-6), which in turn downregulates the cytotoxic T-cell (CTL) responses in these tumours.

**Material and Method:** Using genechip arrays, we analyzed the mRNA expression of all the membrane associated CEACAM family members in CD138+ cells from the bone marrow of 140 multiple myeloma patients and compared it to the healthy donors. Breast cancer cell lines, KS and MCF7, were also analyzed for their surface expression of CEACAM-6 using FACS staining. Inhibition of CEACAM-6 with either blocking monoclonal antibody or via siRNA-mediated gene knockdown was carried out to investigate the effect of CEACAM-6 expression on the reactivity of antigen-specific CD8+ T cell clones against the tumour. For the in vivo model, NOD/SCID mice were engrafted with the CEACAM-6 positive KS breast cancer cells. After tumour establishment, these mice received an adoptive transfer of human tumor antigen-specific T cell clone, with or without the administration of CEACAM-6 blocking antibody and were analyzed for tumour growth.

**Results and Discussion:** CEACAM-6 was found to be overexpressed on freshly isolated myeloma cells from patients and also in breast cancer cell lines − KS and MCF7. Blocking of CEACAM-6 on the surface of myeloma cells completely restored the reactivity of autologous CD8+ T cells against the malignant plasma cells. Moreover, siRNA-mediated knockdown of CEACAM-6, as well as its inhibition by specific mAb, strongly increased the cytokine secretion and antigen-specific lysis of CEACAM-6 positive breast cancer cells by the antigen-specific CTL clone. In the NOD/SCID mouse model, CEACAM-6 blockade using the mAb resulted in a marked reduction in tumour volume of the xenotransplanted breast tumour in comparison to the adoptive transfer alone.

**Conclusion:** Our findings suggest an immunosuppressive role for tumour-associated CEACAM-6, which is mediated through the downregulation of CTL activity. Therapeutic targeting of CEACAM-6 in epithelial as well as hematological tumours might be an attractive clinical approach – either on its own or in conjunction with immune cell transfer therapy.

**[1102] In Vitro Enhancement of NK Cell Activity of Metastatic Melanoma Patients With IFN-α Alone as Opposed to Its Combination With 13-cis Retinoic Acid Is Associated With Modulation of NKGD2 and CD161 Activating Receptor Expression**

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**Introduction:** Melanoma (MM) patients with advanced disease have immunoparesis in immune response, including decreased NK cell activity. As MM is an immunogenic tumor immunomodulating agents, such as IFN-α have been included in the treatment. Considering its limited therapeutic effect, its combination with other immunomodulating agents including retinoic acid (RA) based on its antiproliferative effect and its immunomodulating effect, have been applied in the treatment of MM. As augmentation of NK cell activity by these agents has not been described in the light of newly defined families of NK cell activating and inhibitory receptors, the aim of this study was to investigate in patient the involvement of these receptors in IFN-α and RA induction of NK cell activity.

**Material and Method:** In this study PBL isolated from 36 patients with metastatic MM in clinical stage IV, were cultivated for 18 hours in RPMI 1640 culture medium (CM), alone, CM supplemented with IFN-α (250 U/mI) and/or 13-cis retinoic acid (10−7 M). NK cell activity was determined using standard radioactive 51-Cr assay. Surface immunophenotyping of CD3+CD16+ NK cells was identified by Flow cytometry.

**Results and Discussion:** NK cell cytotoxic activity was augmented after in vitro treatment with IFN-α alone and in combination with RA, even though only IFN-α induced increase in NKGD2 and CD161 activating NK cell receptor
expression. Contrary, RA treatment increased expression of inhibitory KIR CD158b. The IFN-α obtained increase for CD161 expression was due to its induction on both NK cell subsets while that for NKGD2 only on CD16+Bright subset.

**Conclusion:** Our in vitro predictive pretreatment findings of the enhancing effect of IFN-α on NK cell activity of investigated MM patients is associated with up-regulation of activating NKGD2 and CD161 NK cell receptors, while the lack of RA-associated up-regulation may be due to the increased expression of the inhibitory KIR CD158b NK cell receptor after in vitro RA-treatment on CD16-defined NK cells and their functionally diverse dim and bright subsets. However, enhanced NK cell activity by combination of IFN-α with RA probably follows from up-regulated cytotoxic mediators. In this sense, the obtained data, for a standard immunotherapeutic agent, IFN-α, and especially for RA, give novel insight of their modulation of a new generation of NK cell receptors in augmenting antitumor NK cell activity of investigated MM patients.

**[109] Relationship Between IL-10 and Dendritic Cells in Ovarian Cancer Microenvironment**

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**Background:** Progression of malignant tumors decreases number of dendritic cells (DC). Many studies showed that IL-10 influence the differentiation, maturation and DC functions in vitro. The aim of the study was evaluation of IL-10 levels in the plasma and peritoneal fluid (PF) of ovarian cancer patients. Subsequent aim was evaluation if there is a correlation between the IL-10 concentrations and the percentage of myeloid (M) and lymphoid (L) DC. Plasma and PF IL-10 concentrations were analyzed in relation to FIGO stage, histopathological grade and type of the ovarian cancer.

**Material and Methods:** The study group consisted of 104 women with histologically confirmed ovarian carcinomas. The reference group consisted of 34 women with serous cyst of the ovary. IL-10 levels were analyzed using a sensitive enzyme-linked immunosorbent assay (ELISA). MDC and LDC were estimated by flow cytometry.

**Results and Discussion:** Our study showed, that both the PF and the plasma IL-10 levels in women suffering from ovarian cancer were significantly higher than those in patients with benign disease. Moreover, the PF IL-10 levels were significantly higher in women with III/IV FIGO stages than in patients with the stage II of ovarian cancer. Concentrations of IL-10 correlated with the histological type and with the degree of differentiation of cancer. What’s more, concentrations IL-10 both in the plasma as well as in the PF correlated with the FIGO stages of ovarian cancer classification. Interestingly also observation seems to be the highest concentrations of IL-10 and the highest percentage of lymphoid DC (0.92% and 0.97%) in the PF of women with FIGO III and IV ovarian cancer stages. Moreover, higher IL-10 levels, as well as the significantly higher LDC percentage were found in patients with G3 ovarian cancers than in those with G2 degree (1.02% and 0.47%). The concentration revealed negative correlation with percentage of peripheral blood MDC and LDC. Furthermore, a positive correlation between PF IL-10 concentration and LDC percentage was found.

**Conclusion:** High concentrations of IL-10 and increased percentage of lymphoid DC in the PF of women with advanced ovarian cancer, found in our study, may affect the development of the immune response. A statistically significant relations between the plasma IL-10 concentrations in women suffering from ovarian cancer and the peripheral blood MDC and LDC percentages, suggest the influence of IL-10 on the percentage of dendritic cells.

**[110] Expression of Chemokine Receptor CCR7 in Oral Squamous Cell Carcinoma With and Without Cervical Metastasis**

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Cervical lymph nodes metastasis has been highlighted as the main indicator of both worse prognosis and poor survival in oral squamous carcinoma cell (OSCc). Chemotactic interaction between tumor-expressing CC-chemokine receptor 7 (CCR7) and its ligand CCL21 is based on chemotactic mechanism. This study aimed to evaluate and compare CCR7 protein immunoeexpression in tongue and floor of mouth primary squamous cell carcinoma (SCC) and in its respective cervical lymph nodes in patients with and without cervical metastasis. A retrospective and descriptive study was conducted using paraffin embedded samples from both the tumor and cervical lymph nodes from patients diagnosed at the National Cancer Institute in 2001. Clinical and pathological data were registered. Semi-serialized H&E-stained sections and immunohistochemical reaction using anti-cytokeratin AE1/AE3 antibody were performed in metastasis-free cervical lymph nodes. Based on these results, the whole study population (41 patients) was separated into two groups: without metastasis (WM) and with metastasis (WM). CCR7 immunoeexpression in tumors and lymph nodes was evaluated and correlated with collected data. Semi-serialized H&E-stained and pan-CCK AE1/AE3 immunostained sections from 29 patients (WOM) showed 5 (17.2%) cases of cervical lymph nodes metastasis. Thereafter, WOM group (n = 24) and WM (n = 17) were defined. There was a significant association between pathological stage (p < 0.001) and cervical metastasis. A trend for an association with CCR7 expression in tumors was noted in relation to cervical metastasis (p = 0.058) and tumor thickness (p = 0.051). In the WM group, there was a significant worse global survival in non-alcoholic patients (p = 0.0001), non-smokers patients (p = 0.0002), showing clinical T-stage III and IV (p = 0.004). In the WM group, a worse global survival was verified in patients with a family history of cancer (p = 0.0075) and with recurrence (p = 0.014). A significant disease-free survival was observed only in the WM group, with a worst survival forming-alcoholic patients (p = 0.0118). Occult metastasis was a common event in tongue and floor of mouth SCC. Lymph nodes sectioning associated with pan-CCK AE1/AE3 reaction were found to be an important complementary tool in lymph nodes metastasis detection. Chemokine CCR7 higher immunoeexpression in tumors of patients with cervical metastasis suggests its potential role prognosis biomarker to be confirmed with further investigations.

**[111] In Vitro Activation of NK Cells From Regional Lymph Nodes of Melanoma Patients With IL-2 and IL-15**

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**Introduction:** NK cells are population of innate immune system and play an important role in antitumor immune defense. Since regional lymph nodes (LN) represents the first barrier to malignant cell invasion, the aim of this study was to investigate in melanoma (MM) patients, the ability of two cytokines, IL-2, currently used for MM treatment, and IL-15 which is under intensive clinical investigation and is essential for NK cell maturation, to modulate NK cell antitumor activity of scarcely investigated NK cells from regional LNs.

**Material and Method:** Mononuclear cells were purified from 50 regional LNs of MM patients and cultured for 72 h and 7 days in cell culture medium RPMI 1640 (CM) alone, CM with 200 U/ml IL-2 and CM with 25 ng IL-15. NK cell cytotoxicity was determined by standard 4 h Cr release assay, while perforin (PRF) IRNA level was estimated by r-PCR. Expression of several NK cell receptors (RCs) was analyzed after 7 day in vitro treatments on CD3+ CD56+ NK cells by flow cytometry.

**Results:** Both cytokines induced significant in vitro enhancement of NK cell cytotoxicity against K562 target tumor cell of tumor-infiltrated (LN+) and not-infiltrated (LN-) LNs after 72 h of cultivation. Alongside with the induction of NK cell cytotoxicity, the transcription of IRNA for cytotoxic mediator PRF was significantly induced by each cytokine compared to control CM treatments. After longer 7 day cultivation, the enhancement of NK cell cytotoxicity persisted together with significant increase in expression of activating NKGD2 and cytotoxic CD16 Rcs, and CD69 early activation antigen on gated CD3+ CD56+ NK cells from LN+ and LN−, induced by both cytokines. The analysis of inhibitory KIR RCs showed increase in CD158b KIR expression on NK cells from LN+ and LN− by both cytokines. The expression of other investigated KIR CD158a was induced by IL-15 only on NK cells from LN+. The comparison of the cytokine induced NK cell cytotoxicity with respect to CM after 7 day in vitro treatments, revealed the significantly lower increase in NK cell activity obtained with IL-15 in LN+ compared to LN−, which is probably due to IL-15 induction of CD158a KIR that may act suppressive on NK cell cytotoxicity of LN−.

**Conclusion:** IL-2 and IL-15 induce in vitro activation of NK cell from LNs regardless the LN tumor infiltration probably by enhancing PRF transcription and expression of NKGD2 and CD16 activating Rcs on NK cells.
In Vitro Generated Human Dendritic Cells Exposed to a Potent Maturation Melanoma Cell Lines Lysate Show a Tolerogenic-like Phenotype and Functionality When Exposed to Dexamethasone Prior to the Maturation Stimuli

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Background: The available evidence shows that melanoma is an immunogenic tumor to which we can respond with immunotherapy. Dendritic cells (DCs) play a key role in all immune responses, both innate and adaptive. Their main function is to activate T cells, but they are also important in the induction and maintenance of tolerance to self in steady state. Recently, we demonstrated the effectiveness of a DC-based immunotherapy for improving long-term survival in patients with late-stage melanoma using an allogeneic melanoma cell lysate as a potent maturation stimulus. Suppression of DCs maturation and function has been implicated in the immunosuppressive effects of glucocorticoids by reprogramming them to so-called ‘tolerogenic dendritic cells’ (tolDCs) which can elicit a state of hypo-responsiveness in T cells and induce formation of regulatory T (Treg) cells. So, we aim to study the effect of the extensively used glucocorticoid dexamethasone on the immunogenic properties of the DCs to activate the adaptive immune system as a clinically effective DCs-based immunotherapy protocol.

Materials and Methods: Monocytes were incubated for 3 days in the presence of IL-4 and hGM-CSF, stimulated at day 2 with a melanoma tumor lysate or lipopolysaccharide and harvested at day 3. For the tolDCs protocol, dexamethasone was used twice (day 1 and 2). Nine hours later maturation stimuli were added. Maturation markers were tested by flow cytometry. Cytokine production was performed by ELISA and by FlowCytomix® Technology. The phagocytosis capacity was determined by the incorporation of dextran-FITC and Ovalbumin-AlexaFluor488. The proliferation induction was evaluated by CFSE dilution assay. T cell (CD4+IFN-γ+) and T cells (IL-17+CD4+) populations were determined by flow cytometry.

Results: We here show that an early suppressor stimulus elicits a semimature phenotype between the mature (tolDCs) and immature (DCs) DCs, secreting lower levels of proinflammatory and high levels of a regulatory cytokine, but without altering their phagocytosis capability. tolDCs also repress T cell proliferation and differentiation to effector T helper profiles. Conclusion: The use of an extensively used glucocorticoid like dexamethasone could affect the capacity of newly differentiated monocyte-derived DCs to mature and function when affronted with an immunogenic tumor, thus affecting the activation of a correct adaptive anti tumor immunity.

Histidine-rich Glycoprotein Regulates Macrophage Differentiation

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Introduction: The plasma proteins Histidine-rich glycoprotein (HRG) has been implicated in the regulation of tumor growth and vascularization. HRG-deficient mice (Tugues et al., Can Res, in press), challenged with fibrosarcoma or pancreatic carcinoma grow larger tumors, which metastasize more. The tumors were more hypoxic, necrotic and less perfused in hrg−/− mice compared to wild type, indicative of enhanced vessel abnormalization. HRG-deficiency was associated with a suppressed anti-tumor immune response, with increased infiltration of M2 macrophages on the one hand, and decreased infiltration of dendritic cells and cytotoxic T cells on the other. We have previously shown that HRG’s effect on the tumor microenvironment is mediated by inflammatory cells (Rolny et al., Can Cell 2011, 19:31–44). We now investigated the mechanisms whereby HRG affects monocyte/macrophages.

Results and Discussion: To study HRG’s effect on macrophages we used the monoblastic cell line U937, which differentiates towards the monocyte/macrophage lineage after treatment with vitamin D3 (vitD3) or to the granulocytic lineage after treatment with all-trans retinoic acid (ATRA). Both vitD3 and ATRA induced cell cycle arrest, but only the vitD3 arrest was promoted by HRG. Moreover, HRG further stimulated the upregulation of vitD3-induced expression of CD14 (a marker mainly for macrophages), but suppressed the ATRA-induced expression of CD203 (mainly found in granulocytes). Indeed, treatment with HRG in the presence of vitD3 resulted in elevated expression of FcγRI, CD11c and interferon γ; a signature that defines a more inflammatory macrophage phenotype. Conversely, HRG down regulates the expression of IFN-γ by blocking the early differentiation of monocytes to macrophages, and this regulation is exerted in a specific window during this process and accompanied by expression of proinflammatory genes.
Is It Really Safe the Wait and See Policy After Combined Modality Treatment in Anal Canal Carcinoma

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Methods: 347 patients with the diagnosis of locally advanced rectal cancer were treated preoperatively between 2000 and 2010. The clinical complete response rate to preoperative therapy was 34%. All patients underwent resection subsequent to preoperative therapy regardless of response. The pathologic complete response rate among all patients was 22%. Sphincter resection subsequent to preoperative therapy and we analysed the feasibility and safety of a wait-and-see policy.

Background: Some patients undergo apparent complete tumor regression after preoperative combined modality therapy, and controversy exists about the proper management of these patients. It has been proposed that such patients should simply be observed and not undergo resection. The purpose of this study was to determine the significance of clinical complete response to preoperative combined modality therapy and we analysed the feasibility and safety of a wait-and-see policy.

Results: With a median follow up of 69 months, at 5 years local control, distant recurrence free survival, disease specific survival and overall survival were 92, 75, 80 and 70% respectively. 95 patients (27%) had recurrences through out our follow up, 27 were locoregional recurrences and 48 failed distantly. The pathologic complete response rate among all patients was 22%. Sphincter preservation and abdominoperineal resection were preformed in 65% and 35% of the patients respectively. Total mesorectal excision was accomplished in 51% of the patients. 27% were given postoperative chemotherapy. Survival estimates were obtained using Kaplan–Meier curves and Cox proportional hazards model and logistic regression odds ratio were used in the multivariate analysis.

Conclusions: In our experience clinical stage category of disease has a significant impact in all analyzed outcomes, patients who received FDT had an improvement in OS and CSS. Results for more advanced tumors (IIb) remain poor, and require strategies to improve outcome. Higher doses or better treatment compliance may be required. We discourage planned treatment gaps.

Impact of Radiotherapy on PBMCs DNA Repair Capacity – Use of a Multiplexed Functional Repair Assay

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Introduction: Radiation therapy is an essential part of cancer treatment as about 50% of patients will receive radiation at least once. Significant broad variation in radiosensitivity has been demonstrated in patients. About 5−10% of patients develop acute toxicity after radiotherapy. Therefore there is a need for the identification of markers able to predict the occurrence of adverse effects and thus adapt the radiotherapy regimen for radiosensitive patients.

As a first step toward this goal, and considering the DNA repair defects associated with hypersensitivity radiation syndromes, we investigated the DNA repair phenotype of cancer patients receiving radiotherapy. More precisely, we used a functional repair assay on support to follow the evolution of the glycosylases/AP endonuclease activities of PBMCs extracts of a series of patients during the time course of radiotherapy.

Materials and Methods: For each patient, we collected one PBMCs sample before the first radiotherapy application (S1) and three samples after (S2 to S4) (one day and one week after application 1, and one at the end of the radiotherapy protocol). These four samples have been analysed for 11 donors.

Results and Discussion: Clustering analyses of the results demonstrated a great heterogeneity of responses among the patients. Interestingly, this heterogeneity decreased between S1 and S4 where only 2 classes of patients remained if we except one patient that exhibited an atypical DNA repair phenotype. Furthermore, we showed that repair of several oxidized bases significantly increased between S1 and S3 or S4 (8oxoG, thymine glycol, A paired with 8oxoG), suggesting an adaptation of patients repair systems to the oxidative stress generated by the ionizing radiations.

Conclusion: Our preliminary results provided evidence that the DNA repair phenotype was impacted by the radiotherapy regimen. Further characterization of patients with known repair defects is needed to determine if atypical repair phenotypes could be associated with radiotherapy complications. Finally, correlation with clinical data would be useful to identify the parameters responsible for the stratification of patients in two main sub-classes.

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Pulsed Magnetic Field and Ultraviolet C Radiation – Synergistic Effect During Cellular Ageing

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Background: A wide number of articles related to magnetic field effects have been reported during the last years, mainly to study the possible association with cancer risk. However, very few studies have been made using pulsed magnetic field (PMF) in co-exposure with other physical agents. The aim of this study was to investigate the synergistic effect of PMF and ultraviolet C (UVC) radiation during the aging phenomenon of cultured cells.

Material and Methods: S. cerevisiae cells were cultured on YPD medium. They were exposed to PMF (25 Hz, 1.5 mT, 8 hours/day) during the aging process (40 days). In addition, UVC radiation (50 J/m²) was applied on days 1, 20 and 40 of aging. Then, the surviving fraction was measured by clonogenic assay. Four treatment groups were considered: Unexposed control, samples exposed to PMF, samples exposed to UVC, and samples exposed to UVC+PMF.

Results: The cell population experienced a gradual chronological aging from a 100% of survival in the early stages (day 0) until a survival rate of 15% in the latter stages (day 40). The cytotoxic effect of UVC radiation can be clearly seen that causes a decrease in cell survival, being the surviving fraction 0.01% at day 40 of aging. No significant differences were observed between cells exposed to PMF in relation to its relative non-exposed controls in the final stages. However, the surviving fraction obtained (0.001%) for the group treated with MF+UVC was smaller to that obtained for the group exposed only to UVC (0.01%).

Conclusion: The aging cells are more sensitive to UVC. The exposure of aging cells to PMF (25 Hz, 1.5 mT, 8 hours/day) sensitizes the population against UVC radiation (50 J/m²).

X-ray Irradiation Influences Eph Receptors and Cellular Properties in Human Melanoma Cells

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Introduction: There is experimental evidence that X-ray irradiation influences survival and metastatic properties of tumor cells. On the other hand, metastasis and cellular motility can be modified by EphA2 and EphA3, two members of the Eph receptor/ephrin family of receptor tyrosine kinases. The aim of this study was to analyze whether there is a molecular link between X-ray irradiation, Eph expression, and modification of metastasis-associated cell properties in human melanoma cells.

Material and Methods: We irradiated one pre-metastatic and three metastatic human melanoma cells lines, including one self-generated metastatic variant, with X-rays (5 and 10 Gy). At day 1 and day 7 post irradiation (p.i.) we analyzed cell proliferation, colony formation, adhesion, and migration. Additionally, selected Eph receptors and ephrin ligands were analyzed regarding irradiation-dependent changes in mRNA and protein content. For EphA2 and selected downstream signaling molecules we determined the phosphorylation status, respectively.

Results and Discussion: Irradiation resulted in decreased proliferation and colony formation. Colony formation showed partial recovery at 7 days p.i. with 5 Gy. Regarding cell adhesion, we detected an irradiation-induced increment paralleled by a decrease in migration of Mel-Juso and Mel-Juso-L3 cells and, in part, A375 cells. Thus, we assume that X-rays may act anti-metastatic on the investigated melanoma cells. Expression of the ephrins A1 and A5 generally was very low and after X-ray showed a substantial decrement for ephrin A5 in all cells, but a heterogenous behaviour for ephrin A1. For EphA2 we detected a decrease after irradiation both in expression and activity at 7 days p.i. In contrast, EphA3 was found to be up-regulated in 3 of 4 analyzed cell lines, raising the question, if there is a counter-regulation between EphA2 and EphA3. Analyzing downstream signaling, we detected decreased Src kinase and focal adhesion kinase (FAK) phosphorylation in A375, A2058, and Mel-Juso cells at 10 Gy for Src and both 5 Gy and 10 Gy for FAK at 7 days p.i.

Conclusion: Our findings indicate that irradiation-induced downregulation of EphA2 and upregulation of EphA3 in human melanoma cells is associated with anti-metastatic effects. The observed effects are assumed partly to be mediated by regulation of Src and FAK through EphA2.

Potential Role of Lymphocytes During DNA Damage-induced Pneumopathy

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Introduction: Pneumopathy still represents a limiting side effect in the radiotherapy of thorax-associated neoplasms. It is suggested that radiation-induced injury disturbs the equilibrium of lung resident cells and their interactions with the extracellular matrix and infiltrating immune cells. The contribution of the inflammatory cells in the extensive lung lesions and changes in fibrosis development is controversial and needs to be further elucidated. Aim of the present study was to gain more insight into the role of immune cells in DNA damage-induced pneumopathy by using mouse models of whole thorax irradiation and treatment with the radiomimetic drug bleomycin (BLM).

Materials and Methods: C57BL/6 wild type mice (WT) or immunodeficient Rag-2−/− mice received a single dose of whole thorax irradiation with 15 Gray (Gy), or were treated with intraperitoneal injections of BLM. Immune cells were isolated from lung tissue, spleen and cervical lymph nodes at defined time points and characterized via flow cytometry. Moreover, lung tissue samples were used for histological and immunohistochemical analyses.

Results and Discussion: In WT mice, ionizing radiation and BLM-treatment induced time-dependent changes in leukocyte levels and characteristic alterations in the expression of specific surface molecules and other phenotypic markers (e.g. CD73, FOXP3) on distinct immune cell subsets in lung tissue and in lymphoid organs. Moreover, whole thorax irradiation and BLM treatment led to collagen deposition in the lung tissue of WT mice, whereas no such changes were observed in control mice (0 Gy or PBS treated). Fibrosis development after irradiation was associated with tissue hypoxia and increased levels of TGF-β and α-SMA. Of note, irradiated Rag-2−/− mice showed an increased number of fibrotic foci in the lung 168 days after irradiation, when compared to WT mice.

Conclusion: Our data indicate that, in WT mice, whole thorax irradiation and BLM treatment alter composition and activation/surface expression pattern of immune cell subpopulations in the lung and lymphoid organs, followed by tissue hypoxia and increased collagen deposition in the lung. The intensification of radiation-induced fibrosis in Rag-2−/− mice suggests a role of mature B and T lymphocytes in the suppression of fibrotic changes. The role of specific immune cell subpopulations for DNA damage-induced tissue injury and fibrosis is under current investigation.

Long-term Results Treatment of Nasopharynx Malignant Tumors in Children and Teenagers

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Background: To study long-term results treatment of nasopharynx malignant tumors in children and teenagers.

Materials and Methods: In research we were 74 patients with nasopharynx malignant tumors, age from 0 to 18 years. Histologic undifferentiated cancer revealed in 44 patients, planocellular cancer in 15 patients, lumpsocarcinoma in 6, esthesioneuroblastoma in 7, Hodgkin lymphoma in 1, neuroblastoma in 1. In patients performed clinic-roentgenologic, endoscopic, computer tomography and magnetic resonance tomography examinations. Level spreading of tumour process estimated by TNM: T1N0−M0 − 2 patients; T3N0−M0 − 36; T3N3−M1 − 1; T1N0−M0 − 17 and T4N3−M1 − 6. Main treatment method was combined-radiotherapy with using biodegradable drug VCAP (vincriistol + cyclophasphan + docosorubicin + cycpsylatin) then followed radiotherapy and four time chemotherapy.

There were two radiotherapy methods: in 28 patients (37.8%) radiotherapy performed by method ‘semi-semi’ worked out in National Cancer Center of Uzbekistan and in 46 (62.2%) used standard methods. In standard radiotherapy used 2 gray single dose, total dose was 55–60 gray. In exposure by scheme ‘semi-semi’ radiotherapy carried out multifractionation method with 1.2 gray single dose a day, total dose was on primary area − 66–72 gray, on lypf outflow − 36–40 gray.

Results: Patients controled from 6 month to 4 years. In group of malignant tumor patients with using standard radiotherapy and chemotherapy 3 years survival rate was 60.8%, but using method ‘semi-semi’ tegalnammotherapy survival rate was 89.3%.

Conclusion: Chemio-radio therapy is method of election in treatment of nasopharynx malignant tumors, especially with using radio therapy by scheme ‘semi-semi’.

Analysis of Clinical Manifestations and Diagnostic Signs of Osteosarcoma With Lung Metastases

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Background: To study clinical manifestations and diagnostic signs of pulmonary metastasizing process of osteosarcoma.

Material and Methods: We had analyzed 33 patients with lung metastases osteosarcoma, which had been treated in the National Research Center of Oncology of Uzbekistan, during 2007–2010 years. Females − 19 (58%),
BRAF Mutations in Thyroid Carcinomas Following Childhood Alpha-radioimmunotherapy

Introduction: Exposure to ionizing radiation at young age is known as the strongest risk factor for thyroid carcinoma development, namely papillary thyroid carcinoma (PTC). The genetic alterations associated with PTC are strongest risk factor for thyroid carcinoma development, namely papillary thyroid carcinoma (PTC). The genetic alterations associated with PTC are rarely observed in the same tumor. The aim of this study is to evaluate the hotspot BRAFV600E mutation at nucleotide 1559 via immunohistochemistry on paraffin-embedded samples from 35 thyroid cancer, 16 diagnosed by us. Twenty-one PTC tumour samples were screened, through DNA sequencing of the PCR-amplified exon 15, for the hotspot BRAFV600E mutation at nucleotide. Screening of Ras mutations and RET/PTC rearrangement. 

Results and Discussion: We detected the presence of the BRAFV600E mutation in 4 out of 21 post-tinea capitis irradiation PTCs (19.0%). The hotspot BRAFV600E mutation at nucleotide 1559 was dose-dependent, with full recovery of both white blood cells and platelets. The higher activity was probably closer to the MTA. 211At-BR96 radioimmunotherapy showed promising therapeutic activity in a tinea capitis scalp irradiated setting.

Conclusion: The target organ of osteosarcoma metastatic spread is lung and bone, and metastases are usually detected by both lung and bone, featuring osteosarcoma is multiple affecting of lung.

Material and Methods: We have clinically observed and followed 1367 individuals irradiated in childhood for lipoma capitis scapula eruption from which 35 had thyroid cancer, 16 diagnosed by us. Twenty-one PTC tumour samples were screened, through DNA sequencing of the PCR-amplified exon 15, for the hotspot BRAFV600E mutation at nucleotide. Screening of Ras mutations and RET/PTC rearrangement. 

Results: Complete response was seen in 5/6 rats given either 2.5 or 5 Mbq 211At-BR96 compared to 1/6 rats in the control group. Metastases were detected in all groups after treatment. Body weight decreased after treatment with a delayed recovery in the group given the higher activity. Myelotoxicity was dose-dependent, with full recovery of both white blood cells and platelets. The higher activity was probably closer to the MTA.

Conclusion: 211At-BR96 radioimmunotherapy showed promising therapeutic activity in a tinea capitis scalp irradiated setting.

Survival Progeny Derived From Irradiated Parental Cells – Phenotype Analysis and Correlation With Tumour Progression

Introduction: Colorectal cancer (CRC) is one of the most common tumors among population and ionizing radiation (IR) is used as first line of treatment. However, the local recurrence, second malignances and metastasis remain a problem of this therapy. Irradiated-tissue-microenvironment could modulate the survivor progeny, allowing potential malignant advantages for tumor repopulation at later times. The purpose of this study was to analyze events related with the tumorigenic potential in survivors CRC cells submitted to irradiation.

Material and Methods: We used Caco-2, HT-29 and HCT-116 cells as CRC model. Cells were irradiated with 5 Gy in Cs137 irradiator. After 24 and 48 hours, cells were trypsinized and maintained in culture to form colonies. Cell morphology was analyzed by phase contrast. Expression and localization of junctional complex proteins and intermediate filaments were monitored by immunofluorescence and immunoblotting. Activations of the actin cytoskeleton was monitored by using conjugated F-actin-TRITC and confocal microscopy. Wound healing assay ensures the cell migration; radioresistance was monitored by clonogenicity and Caspase-3 assay and the anchorage independent growth were analyzed by agarose assay. Anti apopotic protein survivin were monitored by immunoblotting.

Results and Discussion: The survival progeny derived from irradiated parental cells Caco-2, HT-29 and HCT-116 showed an aberrant morphology, with lamellipodium and philopodium projections, as compared with control cells. Confocal microscopy analysis showed heritable aberrations in actin cytoskeleton organization and disorganization of cell contacts as seen by the internalization of E-cadherin and β-catenin in all cell lines progeny. In addition, reduced E-cadherin in Caco-2, HT-29 and HCT-116 and elevated β-catenin expression in HT-29 cells was also observed by immunoblotting.
 Increased expression and reorganization of vimentin filaments was observed only in HT-29 by immunoblotting and immunofluorescence. A higher migratory potential was observed in the HT-29 progeny, as compared with control cells. Clonogenic survival and viability analyses showed that Caco-2 were more radioresistant than HT-29 and HCT-116, respectively, but only HT-29 progeny showed an increase of anchorage independent growth in agarose assay. Interestingly, an increase of survivin expression was observed by immunoblotting in the HT-29 and Caco-2 progenies, but not in HCT-116 cells.

Conclusion: The results suggest that IR induces heritable phenotypic alterations that are correlated with a more aggressive potential in the survival progeny derived of parental irradiated cells, which could contribute to the development of refractory tumors and metastasis. More studies are in course to elucidate this hypothesis and find radioresistance biomarkers.

1225 Association Between Variants in the VEGF Gene and Distant Metastases in Postmenopausal Breast Cancer Patients

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Background: Vascular endothelial growth factor (VEGF) is essential for tumor angiogenesis and metastatic spread. The purpose of the present study was to analyze the role of VEGF polymorphisms and haplotypes for the development of metastatic progression in postmenopausal women with breast cancer.

Material and Methods: A total of 584 postmenopausal breast cancer patients from the Austrian TIGER study ("tumor of breast tissue: incidence, genetics, and environmental risk factors") were eligible for analysis. The occurrence of distant metastases was evaluated in regular follow-up examinations. Seven variants in the VEGF gene were selected and genotyping was done by a 5'-exonuclease assay (TaqMan). Haplotypes and linkage disequilibrium were determined using the Haploview program. Statistical analysis was performed using SPSS 18.0 for Windows.

Results: Within a median follow-up time of 77 months (range 0–121 months), 122 (21%) patients developed distant metastases. In a Kaplan–Meier analysis, carriers of the −634G allele developed distant metastases (p = 0.027) and in additional Cox regression analysis, the hazard ratio for distant metastases was 0.69 (95% CI 0.52 to 0.92, p = 0.012). Furthermore, the CCCC haplotype formed by 5 polymorphisms upstream of the coding sequence including the −634G>C polymorphism showed a significant association with distant metastases (HR 0.655, 95% CI 0.487 to 0.882; p = 0.004). In a multivariate analysis including tumor stage, tumor grade, initial lymph node involvement, hormone receptor status and HER2/neu status as potential confounders, the CCCC haplotype remained a significant predictor of distant metastases (HR 0.614, 95% CI 0.416 to 0.906; p = 0.014).

Conclusion: We conclude that genetic variants in the gene for VEGF may influence the risk of the development of distant metastases in postmenopausal breast cancer patients.

1226 Role of Polymorphisms in the ERCC2 Gene in the Development of Severe Side Effects After Radiotherapy of Prostate Cancer Patients

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Background: DNA repair genes are involved in the repair of genotoxic damage induced by ionizing radiation. In this study we examine the dynamics of changes and the sources of ROS and RNS as well as strand breaks in DNA and cell death in two human cell lines irradiated with a single X-ray dose.

Materials and Methods: Human leukemic K562 and HL-60 cells were exposed to 12 Gy of ionizing radiation. Intracellular ROS and nitric oxide were assayed using 2′,7′-dichlorofluorescein (DCF) and 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM), respectively. Mitochondrial complex I was inhibited by rotenone. The kinetics of DNA strand break rejoining were measured by comet assays and cell death was quantified using Annexin-V–propidium iodide staining and flow cytometry.

Results: Reactive oxygen and nitrogen species were measured at different time points after exposing cells to X-radiation. Irradiation caused an immediate increase of about 60% in the level of ROS, followed by a gradual decrease during the next 4 h after which a second increase persisting longer than 24 h was observed in both cell lines. The dynamics of the changes in ROS and RNS levels were similar in both cell lines, but K562 cells showed a higher level of ROS in the second peak. The increase in these radicals was correlated with an increased level of DNA strand breaks and of cell death in both cell lines. To establish the source of the increased ROS, we used rotenone to inhibit the mitochondrial complex I. The initial increase of ROS was not inhibited by rotenone suggesting that it was induced directly by irradiation, but the late increase was inhibited by rotenone in K562 but not in HL60 cells.

Conclusions: Exposure to ionizing radiation induces long-lasting changes in the levels of reactive oxygen and nitrogen species in cells and increases cell death. The increase of these radicals observed 24 h after irradiation results from cellular metabolic processes, and the signaling pathways involved are different in different cell types. The work was financed by Polish Ministry of Science and Higher Education grant MN 516497839.

1227 Increased Levels of Reactive Oxygen and Nitrogen Species in Cells Exposed to Ionizing Radiation

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Background: Free-radical products are the main cell-damaging compounds induced by ionizing radiation and include reactive oxygen (ROS) and reactive nitrogen (RNS) species. ROS are formed as a result of radiolysis of water as well as by a number of intracellular processes. Metabolically-generated secondary radicals can act as damaging agents leading to death or induction of genomic instability, and have been shown to play a role in cancer development.

Materials and Methods: Human leukemic K562 and HL-60 cells were exposed to 4Gy of ionizing radiation. Intracellular ROS and nitric oxide were assayed using 2′,7′-dichlorofluorescein diacetate (DCF) and 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate (DAF-FM), respectively. Mitochondrial complex I was inhibited by rotenone. The kinetics of DNA strand break rejoining were measured by comet assays and cell death was quantified using Annexin-V–propidium iodide staining and flow cytometry.

Results: Reactive oxygen and nitrogen species were measured at different time points after exposing cells to X-radiation. Irradiation caused an immediate increase of about 60% in the level of ROS, followed by a gradual decrease during the next 4 h after which a second increase persisting longer than 24 h was observed in both cell lines. The dynamics of the changes in ROS and RNS levels were similar in both cell lines, but K562 cells showed a higher level of ROS in the second peak. The increase in these radicals was correlated with an increased level of DNA strand breaks and of cell death in both cell lines. To establish the source of the increased ROS, we used rotenone to inhibit the mitochondrial complex I. The initial increase of ROS was not inhibited by rotenone suggesting that it was induced directly by irradiation, but the late increase was inhibited by rotenone in K562 but not in HL60 cells.

Conclusions: Exposure to ionizing radiation induces long-lasting changes in the levels of reactive oxygen and nitrogen species in cells and increases cell death. The increase of these radicals observed 24 h after irradiation results from cellular metabolic processes, and the signaling pathways involved are different in different cell types. The work was financed by Polish Ministry of Science and Higher Education grant MN 516497839.

1228 Reactive Oxygen Species May Play a Role in Ionizing Radiation-induced Bystander Effects and Regulation of mRNA Levels by MicroRNAs

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Background: Cellular responses to ionizing radiation depend on direct effects and also on intercellular communication manifested as bystander effects. We have shown previously that the transcriptomes of directly-irradiated and bystander cells are highly different, and that bystander effects depend strongly on oxidative processes in the cells. This study shows an influence of mutual interactions between irradiated and unirradiated cells at the level of reactive oxygen species (ROS) and presents analyses of transcript levels and their changes in irradiated human cells.

Materials and Methods: Human M45 melanoma cells or HDF fibroblasts were exposed to 4 Gy of ionizing radiation and co-cultured with unirradiated cells. Intracellular ROS were assayed using 2′,7′-dichlorofluorescein diacetate, 4-amino-5-methylamino-2′,7′-dichlorofluorescein diacetate, microneuros and apoptosis frequencies by fluorescence and phase-contrast microscopy in DAPI-stained cells, mRNA and mRNA levels with Affymetrix HG-U133A and
An in Vivo Evaluation of the Potential of 188Re-PEI-MP for Therapy of Bladder Carcinoma and 99mTc-PEI-MP for Diagnosis of Cancer

**Introduction:** The development of water-soluble polymers such as PEI-MP

**Agilent SurePrint G3 Human v16 miRNA microarrays, and the level of 8-oxoG in RNA by HPLC.**

**Results and Discussion:** Irradiation of Me45 and NHDF cells followed by co-cultivation with unirradiated cells induced an increase in the levels of ROS, mucin, and apoptosis in both the irradiated cells and unirradiated neighbours. The levels of ROS, mucin and apoptosis in irradiated Me45 cells were reduced when they were co-cultured with unirradiated NHDF cells, suggesting a protective influence. Transcripts which were up-regulated after irradiation in Me45 cells contained significantly higher numbers of motifs targeted by miRNAs than transcripts which were down-regulated or unchanged. The level of 8-oxoG in RNA was increased in irradiated cells. Together, these findings are consistent with the model that up-regulation of transcript levels in irradiated cells results from partial disruption of interactions of microRNAs with mRNAs, possibly by damage to RNA by reactive oxygen species.

**Conclusions:** Irradiated cells can be partially protected by neighbouring unirradiated fibroblasts. This mutual communication between irradiated and unirradiated cells can influence the cellular production of reactive oxygen species. The changes in transcript levels observed in cells exposed to irradiation may at least in part result from disruption of interactions of microRNAs with mRNAs caused by damage to RNA by reactive oxygen species.

**Material and Method:** Cytotoxicity of PEI-MP was investigated in bladder carcinoma cell line (CRF-1472) using the MTT test for different concentrations of PEI-MP (1 μM to 1000 μM) and incubation times (24, 48, 72 and 96h). Radiochemical purity of 188Re-PEI-MP and 99mTc-PEI-MP was achieved using ascending microchromatography. The in vivo studies were performed after the approval by Ethics Committee of the Faculty of Medicine, University of Coimbra. It was used six groups of Balb/c nu/nu mice: four normal groups injected with Na188ReO4 (n = 18) 188Re-PEI-MP (n = 17), Na99mTcO4 (n = 10) and 99mTc-PEI-MP (n = 10), respectively, two with bladder carcinoma xenotransplanted with Na188ReO4 (n = 16) and 188Re-PEI-MP (n = 12), respectively. When reached the appropriate volume, Na188ReO4, 188Re-PEI-MP, Na99mTcO4 or 99mTc-PEI-MP were administered by an intravenous injection in the tail vein (22–37 Mbq), with the animal anesthetized and previously placed on the gamma camera detector. Immediately, a dynamic acquisition followed, with a 128x128 matrix for 10 min (20 frames, 30 seconds).

**Results:** The SCP could be analyzed by phase contrast imaging of shapers up to 60 days after treatment using a Spheroid-Based Test platform. Determination of the SCD50 demonstrated FaDu-shapers to be more sensitive to external irradiation alone than SAS-shapers. Treatment with un conjugated C225 prior to external irradiation showed no radio-sensitizing effect in FaDu- and SAS-shapers. Competitive radioactive binding studies revealed that the affinity of C225 to EGFR is not affected by conjugation with the radionuclide 90Y. Also, similar penetration kinetics of unconjugated- and 90Y-conjugated C225 into shapers were detected. Combined treatment of external irradiation with 90Y-conjugated C225 (1 μg/ml, 24 h) resulted in a clear therapeutic benefit as reflected by a pronounced reduction of the SCD50 from 16 G to 9 G for SAS-shapers and a complete loss of regrowth for FaDu-shapers.

**Conclusion:** The SCP and the SCD50 could be established to monitor therapeutic efficacy in shapers of combined treatment using external and internal irradiation. Tumor shapers are a suitable in vitro model to monitor therapy-relevant properties of radionuclide-conjugated therapeutic antibodies. The combination of 90Y-conjugated C225 with external irradiation is a promising therapeutic strategy.

**Supported by the German Federal Ministry of Education and Research (BMBF) through grant 02NUK006.**

**[112]** The HDAC Inhibitor Vorinostat (SAHA) Down-regulates C-FLIP and Sensitizes Human Non-small Cell Lung Carcinoma Cells Lines to Ionising Radiation

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**Background:** Non-small cell lung cancer (NSCLC) patients have a poor prognosis with a 5-year survival characterized by external and a significant clinical contribution to the treatment of NSCLC. Strategies to increase the therapeutic index of radiation are sought to improve treatment outcomes. c-FLIP is an anti-apoptotic protein that blocks death receptor-mediated apoptosis by preventing caspase 8 activation at the death-inducing signalling complex (DISC). We have previously shown that c-FLIP is an inhibitor of apoptosis induced by DNA damaging drugs and that the HDAC inhibitor vorinostat down-regulates c-FLIP protein in various cancer types.

**Results:** Silencing c-FLIP expression enhanced cell death induced by ionising radiation, as detected by PARP cleavage, caspase activity and sub-G1 population analysis by flow cytometry. Clonogenic assays were used to assess long-term survival. Caspase activation was determined using caspase activity assays and Western blotting. The DNA damage response was assessed by γH2AX foci counting and flow cytometry.

**Results:** Silencing c-FLIP expression enhanced cell death induced by ionising radiation, as detected by PARP cleavage, caspase activity and sub-G1 population analysis by flow cytometry.
population. Similarly, pre-treatment with vorinostat sensitized cells to ionizing radiation. γH2AX is a sensitive marker of the cellular response to the presence of DNA double-stranded breaks. γH2AX levels were increased and prolonged in cells in which c-FLIP protein expression was down-regulated.

Conclusion: Our results indicate that c-FLIP down-regulation sensitizes NSCLC cells to DNA damage induced apoptosis. Thus, pharmacological inhibition of c-FLIP using the HDAC inhibitor vorinostat, or other methods is a potential therapeutic approach for enhancing ionising radiation induced DNA damage and cell death.

[1134] Gemicitabine as a Chemoradiosensitiser in Bladder Cancer
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Introduction: Muscle invasive bladder cancer may be treated by cystectomy or radiotherapy-based approaches, but currently we cannot predict which modality is better in an individual patient. Both neoadjuvant chemotherapy and concurrent chemoradiation improve survival rates modestly.

The pyrimidine antimetabolite gemicitabine is an effective radiosensitiser in many cancers, including bladder. We aim to establish predictive biomarkers for response to gemicitabine and use this information to tailor therapy accordingly.

Previously, we showed that patients with low tumour MRE11 expression by immunohistochemistry had poorer survival following radiotherapy alone. MRE11 knockdown in bladder cells causes a two-fold increase in sensitivity to gemicitabine. Therefore, we hypothesised that low MRE11 expression might benefit from concurrent gemcitabine and radiotherapy. However, sensitisation could also be influenced by alterations in gemicitabine-metabolising enzyme expression.

Materials and Methods: RT112, CAL29, T24 and VMUCB1 bladder cancer cell lines were treated with gemcitabine for 24 h prior to replating at appropriate cell density and irradiated with 0–8 Gy for clonogenic survival assay. Cells were stained with propidium iodide for flow cytometry analysis of the cell cycle. Western blots and qPCR were performed to establish protein and mRNA levels, respectively.

Results and Discussion: Gemicitabine radiosensitised a panel of four bladder cancer cell lines after 24 h exposure in the nanomolar range (IC50 8.5 nM – 35 nM). Cell lines that were conditioned to become resistant to 10 μM gemicitabine showed striking downregulation of deoxyxycytidine kinase (dCK), an enzyme crucial for the conversion of gemicitabine from prodrug to its active form. Reduction in mRNA levels of dCK exceeded 50% and protein levels were undetectable by western blot. This is in contrast to cells treated with gemcitabine for 24 h, where a small increase in dCK protein levels was observed.

Radiosensitisation corresponds to an accumulation of greater than 85% cells in S phase, but the gemcitabine resistant lines do not accumulate in S phase (S phase population less than 35%, equivalent to untreated parental cells) and resistant cells no longer display radiosensitivity. However, overexpression of dCK in resistant cell lines is sufficient to reestablish cells to gemcitabine and reestablish its radiosensitising effects.

Conclusions: Gemicitabine was an effective radiosensitising agent in a panel of bladder cell lines and cells with acquired resistance displayed downregulation of dCK. Overexpression of dCK was sufficient to overcome resistance to gemcitabine, indicating that this enzyme is key in defining how cells respond to the drug. We shall now determine whether dCK downregulation impacts on gemicitabine radiosensitisation in cells expressing low levels of MRE11.

[1135] The Influence of p53 in Iodide-131 Treatment Efficacy in Hepatocellular Carcinoma
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Background: Hepatocellular carcinoma (HCC) is the most common primary liver malignancy with a rising incidence worldwide, representing the third most frequent cause of cancer death in the world. Overall survival at 5 years for patients with HCC is approximately 2–10%, so it is urgent to study and develop new therapeutic strategies. Radiation therapy is now commonly accepted as one of the most effective form of cancer treatment. The success of ionizing radiation for treating cancer depends on its ability to selectively kill the tumor cells. One of the key molecules involved in a cell’s response to ionizing radiation is the tumor suppressor gene, p53. This work aims to investigate the iodine-131 therapeutic efficacy in three human HCC cell lines which differ in the p53 expression.

Methods: To perform this studies we used three different human HCC cell lines: HepG2 (wp53), Hep3B2.1−7 (p53 null) and Huh7 (mp53), which were subjected to internal and external radiation with iodine-131. To evaluate cell viability clonogenic assays were performed. Flow cytometry was carried out to examine cell viability, oxidative stress, intracellular expression of reduced glutathione, mitochondria membrane potential and cell cycle after irradiation. In order to determine the p53 expression in response to radiation we resorted to the western blot. Real-Time PCR was performed to determine the expression of mRNA of the TP53 gene.

Results: The results show that cells expressing mutated p53 (Huh7), that induces an overexpression of this protein, is the most radiosensitive, because shows a greater reduction in viability. Flow cytometry revealed that the preferential type of death by HepG2, Huh7, and Hep3B2.1−7 cell lines when irradiated with iodine-131 is the late apoptosis/necrosis, with best results obtained for high exposure doses. For all the cell lines studied, there is a general trend of increasing the concentration of reactive oxygen species, as well as, reduced glutathione and mitochondrial membrane potential. The p53 protein induces mostly cell growth arrest in the G0/G1 phase.

For western blot, it was observed that the Huh7 cell line presents higher levels of p53 expression, followed by HepG2 cell line and finally Hep3B2.1−7 cell line. For Real-Time PCR, it was observed that HepG2 and Huh7 cell lines expressed mRNA, unlike to the cell line Hep3B2.1−7.

Conclusion: We conclude that the tumor suppressor gene can be a key factor in response to radiation therapy. The results show that cells expressing mutated p53 are the most radiosensitive, which may result in a more favorable prognosis for tumors that express this gene, data that can be very useful in clinical practice.

[1136] Investigation of the Role of Acid Sphingomyelinase in the Bystander Effects of Breast Cancer Cell Irradiation
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Introduction: Acid sphingomyelinase (ASM) is a member of the Dnase 1 enzyme superfamily that can facilitate the breakdown of sphingomyelin, a major lipid component of mammalian cell membranes to ceramide, a bioactive lipid involved in the activation of cell death signalling pathways. The aim of this study was to investigate the role of ASM in the bystander effects of cellular irradiation.

Methods: (Exp 1) The human breast tumour cell line, T47D-3, was cultured to 80% confluency and seeded to approximately 800,000 cells per flask and incubated overnight to allow cells to attach. Cells were irradiated at using a Varian Clinac 6–100 linear accelerator (Varian Medical Systems Inc., USA) at 0.5, 1.0, 2.0, 4.0 and 8.0 Gy using 6MV X-ray beam at 2Gy/min nominal dose rate. Radiation fields size covered the whole flask, imbedded in a tissue-equivalent phantom to ensure full scatter conditions. Lincac dose output has been verified using ionisation chamber device and standard calibration procedure. A control flask was included to assess any stresses the cells may be under throughout the process. 1mL aliquots of the cell culture medium were sampled at intervals post irradiation and TNF-α and ASM activity was determined using an ELISA kit and reactive oxygen species (ROS) were determined using Electro Paramagnetic Resonance (Bruker Biospin GmbH, Germany). The epithelial colorectal adenocarcinoma cell line, Caco-2, was cultured to 80% confluency, then media from the T47-D-3 irradiated cells was transferred to Caco-2 cells, ASM activity increased to 134% of controls in the 8Gy irradiated media. Cell viability in the Caco-2 cells was reduced to 0.6% in the 8Gy irradiated media indicating total cell death demonstrating the bystander effect. Caco-2 cells incubated for 4 hours with ASM alone showed between 27 and 48% reduction in cell viability. 48 hours post irradiation T47D-3 cells demonstrated ROS was decreased by 20–30% compared to controls and returned to baseline by 72 hours.

Conclusion: This study has demonstrated the bystander effects associated with cellular irradiation and shown that ASM may contribute to these bystander effects. Similarly, ROS may play an important role as a secondary messenger.

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**Clinical Characteristics of Uzbek Patients With BRCA1 Mutated Ovarian Cancer**

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**Background:** BRCA is a new clinical concept attempting to identify groups of patients with constitutional BRCA1 and BRCA2 deficiency. A recent Russian study among 29 patients with BRCA1 and BRCA2 mutation, showed high incidence of metastasis evolution whereas it was uncommon among a group of 48 sporadic ovarian cancer. The aim of our research was to confirm in another population these results.

**Material and Methods:** Histological and clinical characteristics were retrospectively analyzed in 2 BRCA1 and 9 BRCA2 mutated ovarian cancer diagnosed between 2002 and 2010 in Fergana Region. F control group of sporadic cancer patients without familial or personal history of breast or ovarian cancer was identified in the same period with a 2:1 ratio. 92 patients were analyzed (30 patients in the BRCA1, 43 group, and 62 patients in the sporadic group). The mean age was 52 (36–64) and 62 (20–82) years retrospectively. At diagnosis, 63% and 84% were treated for an advanced stage in the BRCA group (stage III: 14 patients, stage IV: 4) and sporadic group (stage III: 34 patients, stage IV: 18). The histological subtype was serous for 60% and 71% of BRCA and sporadic group. At baseline, no visceral metastasis was found in the BRCA group, in contrast with 13% in the sporadic group (liver, lung). During the follow-up 43% of BRCA and 34% of sporadic cancer patients developed metastasis (liver, bone, lung and brain). Platinum sensitivity mean time was 52 months [7–192] in the BRCA group and 30 months [3–53] in the sporadic group (43 and 25 months among advanced stage respectively). The overall survival was 66 months and 37 months for BRCA and sporadic group (50 and 34 months among advanced stage respectively).

**Conclusions:** This study confirm that ovarian cancer patients constitutional BRCA mutations are younger, have longer sensitivity to platinum and better overall survival than sporadic ovarian cancer patients. However we did not confirm that visceral metastasis as a specific BRCaness profile.

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**Clinical Use of HER2 Extracellular Domain as a Marker to Monitor Cancer Status and Predict the Response to Anti-cancer Treatment in Breast Cancer**

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**Background:** We assessed the clinical usefulness of HER2/neu extracellular domain (HER2EDC) as a biomarker for monitoring cancer status and for predicting the efficacy of anti-cancer treatment and prognosis of patients with breast cancer.

**Methods:** Five-hundred and seventy-eight serum samples from 251 patients with breast cancer were examined for the concentration of HER2EDC and conventional tumor markers (CEA, CA15-3, NCC-ST439 and BCA225). The serum levels of HER2EDC were compared with those of conventional tumor markers and with clinical cancer status of the patients. Also, in eighteen patients with HER2-overexpressed advanced or metastatic breast cancer who were treated with trastuzumab, which targets the HER2 molecule, outcomes were assessed to determine whether their serum HER2EDC levels predict the efficacy of treatment they received.

**Results:** The percentage of patients positive for HER2EDC was 15.6%, which was compatible with that for the conventional markers. In patients with HER2-overexpressed advanced or metastatic cancer, the positive rate of HER2EDC (25%) was significantly higher than that of other conventional markers (9.8 to 15.7%). Furthermore, the serum HER2EDC level was strongly correlated with cancer status, with a high progression and regression. When serum HER2EDC levels were compared between HER2-overexpressed patients responding to and those not responding to trastuzumab-containing regimens, the levels were significantly higher in responders than in non-responders (p = 0.003). While the time to progression (TTP) was significantly longer in responders than non-responders (p = 0.003), there was no difference in TTP between patients with HER2EDC-positive cancer and those with HER2EDC-negative cancer (p = 0.175).

**Conclusions:** The results suggest that HER2EDC is not only a useful tumor marker reflecting cancer status but also a biomarker predicting the clinical efficacy of trastuzumab in HER2-positive breast cancer.

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**Tumor-associated Antigens and Their Cognate Autoantibodies as Potential Diagnostic Tool for Breast Cancer Detection**

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**Introduction:** Autoantibodies to tumor-associated antigens can serve as immune diagnostics markers and be an in vivo probe for tumor cell immunogenicity. We reveal by serological plaque assay that in 18 of them have cancer-restricted serological profile. To verify this data we carried out a large-scale allelic screening of 16 recombinant SEREX-antigens using enzyme-linked immunosorbent assay (ELISA) for identification of tumor-related autoantibody profiles in patients with different types of breast tumors.

**Material and Methods:** ELISA was used to detect antibodies against immunogenic recombinant peptides of 16 SEREX-defined TAs in 112 sera from patients with different types of breast cancer (invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma), in 20 sera from patients with benign breast tumors and 35 sera from normal individuals. Positive serological reactivity was defined as an absorbance value greater than 3 standard deviation above the mean value of the healthy individuals’ sera. For evaluation of diagnostic potential of antibodies and their cognate autoantibodies ROC-analysis was applied.

**Results and Discussion:** Antibody frequency to the individual TAs didn’t exceed 21% and didn’t correlate with tumor grade. Significantly higher frequency of antibody response compared to control cohort was shown in breast cancer patients to 6 antigens that didn’t correlate with ER, PR, HER-2/neu and lymph node status of patients. Diagnostic sensitivity of individual antigens was quit low and didn’t exceed 41%, but a combination of 6 from 16 antigens in a single panel had significantly higher sensitivity (70%) and specificity (91%) for breast cancer detection.

**Conclusion:** We characterized new breast cancer associated antigens in large-scale allelic screening and proposed the panel of 6 antigens with high diagnostic potential that could be used for breast cancer associated autoantibody profile detection.

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**Germline BRCA1/BRCA2 Mutations in Breast/Ovarian Cancer Families in Croatia: Identification of Three Novel Mutations**

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**Introduction:** Breast cancer is the most commonly diagnosed cancer in women after non-melanoma skin cancer, and is the leading cause of cancer related deaths in Croatia. Ovarian cancer is in the fifth place, both in incidence and mortality. About 5–15% of breast cancer and 10% of ovarian cancer cases are hereditary, and germline mutations in BRCA1 and BRCA2 genes account for the majority of hereditary breast and ovarian cancers. The purpose of this study was to estimate the incidence and spectrum of pathogenic mutations in BRCA1/2 genes in high risk women in Croatia. This study was funded by The Terry Fox Run 2009 donation and supported by The Terry Fox Foundation and Croatian League Against Cancer.

**Materials and Methods:** 167 candidates from 145 families were scanned for BRCA1 and BRCA2 mutations using High-resolution melting analysis (HRMA), direct sequencing and Quantitative multiplex PCR of short fluorescent fragments (QMPSF).

**Results and Discussion:** We identified 14 pathogenic point mutations in 17 candidates, 9 in BRCA1 and 5 in BRCA2. Of those, 11 have been previously described and three were novel (c.5353C>T in BRCA1, and c.4139_4140dupT and c.1975G>A in BRCA2). No large deletions or duplications involving BRCA1 or BRCA2 genes were identified. No founder mutations were detected for the Croatian population. Two common sequence variants in BRCA1, c.2077G>A and c.4956G>C, were found more frequently in index carriers compared to healthy controls. Haplotype inference showed no difference in haplotype distributions between deleterious mutation carriers and non-carriers in neither BRCA1 nor BRCA2. In silico analyses identified one BRCA1 sequence variant (c.4039A>G) and two BRCA2 variants (c.5986G>A and c.14174G>C) as being significantly different between patients and controls. However, no suggestive results were obtained for our novel BRCA2 variant c.3864_3866delTAA.

**Conclusions:** Combination of QMPSF and HRMA methods provides high detection rate and complete coverage of BRCA1/2 genes. Benefit of BRCA1/2
mutation testing is clear, since we detected mutations in young unaffected women, who will be closely monitored for breast and ovarian cancer.

**[1124] Risk Factors for TEL-AML Fusion Gene and Childhood Acute Lymphoblastic Leukemia in Egypt**


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**Background:** Childhood acute lymphocytic leukemia (ALL) is the most common pediatric cancer. The exact cause is not known in most individual cases, but past epidemiological research has suggested a number of potential environmental and genetic risk factors. This study aimed to: (1) Evaluate environmental (such as pesticides exposure and smoking) risk factors for ALL risk in Egyptian children. (2) Study the associations between polymorphisms in MTHFR, and NQO1 genes and environmental exposures on the risk of ALL in Egypt.

**Material and Method:** The source study, Risk Factors for TEL/AML1 fusion gene and ALL in Egypt (Ezzat S. et al., R03 CA133960), uses a case-control design. Cases (N = 295) were recruited from the Children’s Cancer Hospital, Egypt in the period from 2006 to 2012. Controls (N = 333) were randomly selected from the general population to frequency-match the cases by sex, age and residence. Mothers provided answers to an administered questionnaire about their medical, environmental exposures and occupational history. Blood sample from the mother and the child was drawn to test mutations in studied genes. A TEL-AML fusion gene was tested by forescent in situ hybridization technique. Mutations in MTHFR and NQO1 genes were tested by PCR using the specific primers. Odds ratios (ORs) and 95% confidence interval (CI) were calculated using conditional logistic regression models adjusting for age of the child, maternal age, urban/rural residence and education of parents.

**Results and Discussion:** Comparing cases to controls, it was found that having normal delivery was a protective factor (OR = 0.65, 95% CI 0.45–0.93). Use of fertility medication prior to pregnancy in the index child was associated with increased risk (OR = 2.65, 95% CI 1.24–5.66). Exposure of mothers during pregnancy to Environmental Tobacco Smoke at work or home (other sources than the husband) was associated with increased risk (OR = 16.24, 95% CI 6.24–42.25). Having a mutant allele of MTHFR 2 in mothers was associated with increased risk (OR = 1.38; 95% CI 0.95–2.0). TEL-AML fusion gene was positive in 18.3 of the cases. We did not find any significant difference in the studied environmental and genetic factors in the case-case analysis when comparing cases who have the fusion genes with those who do not have it.

**Conclusion:** Our study showed that normal delivery is a protective factor, while intake of medication that helps in fertility was associated with increased risk. Exposure to ETS during pregnancy was also associated with increased risk. Future studies are encouraged to integrate the use of biomarkers to assess exposures and to elucidate the biological mechanisms for those factors whenever possible.

**[1125] Nine New Alternatively Spliced Isoforms of BRCA2 mRNA – a Clue for Genetic Variants Classification**


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**Background:** Deleterious germ-line mutations in BRCA1 and BRCA2 confer high risk of developing breast and ovarian cancer. However, up to 50% of the sequence changes identified in these two genes are variants of unknown clinical significance (VUS) some of which may compromise gene function. For instance, some may affect gene splicing. There is need to indicate the number of alternative splicing isoforms expressed in humans has been grossly underestimated. Moreover, inter-individual variability exists among healthy individuals. Therefore, to investigate the effect of any VUS on splicing, we first need to characterize non-pathogenic VUS in the gene. In this study, we have evaluated the extent of ‘non-pathogenic’ inter-individual variability. BRCA1 isoforms have been widely studied. Also some very frequent and abundant splicing isoforms have already been described in BRCA2: Δ5, Δ657, Δ12, Δ18 and Δ17–18.

The Splicing Working Group (SWG) of the ENIGMA Consortium has been collecting data from several laboratories on less frequent or less abundant alternative splicing isoforms of BRCA1 and BRCA2. RNA has been collected from LCLs, whole blood and IL/PHA stimulated lymphocytes. Previously, only eight BRCA2 splicing isoforms had been confirmed by Sanger sequencing, including the above mentioned, along with Δ2, Δ5, and intron 20ins. Size calling or RNA-seq results have been collected by the participating labs for 13 more candidate isoforms, but these have not yet been verified by direct sequencing.

**Material and Method:** Our aims are to confirm the existence of the remaining proposed BRCA2 isoforms and to evaluate the inter-individual variability in the expression of all SWG isoforms. We designed isoform specific RT-PCR primer for the new isoforms and analysed RNA from LCLs. For inter-individual analysis, 11 LCLs were used, four carriers of BRCA2 mutations not expected to interfere with splicing, and seven non-carriers.

**Results:** Nine new isoforms were detected and sequenced. We analysed inter-individual variability in the expression of the 12 new isoforms detected by SWG. All LCLs expressed at least seven different isoforms. Three were present in all the LCLs, whereas one was only present in two. The remaining isoforms were expressed by 60–90% of the samples. No particular isoform expression difference was detected between BRCA2 mutation carriers and non-carriers.

**Conclusion:** Characterization of naturally occurring BRCA2 splice variants serves guides both characterization of VUS associated with splicing defects the functions of BRCA2 itself. Four splicing variants are predicted to preserve reading frame and can encode protein products. Others could play functional roles that do not require translation. Future work will be required to determine which splice variants are functional and how they contribute to BRCA2 biology.

**[1146] Assessment of Frequency of the EGFR Mutations According to the Histological Type and Sex of Bulgarian Patients with NSCLC**

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**Background:** Mutations in 18th-21st exons of EGFR gene receptor correlate with positive response to treatment with tyrosine kinase inhibitors (TKI) in patients with NSCLC.

**Methods:** Our cohort consist of 727 tissue specimens from patients with NSCLC. DNA was extracted from paraffin embedded tissues and analyzed for TKI activating mutations in EGFR gene by real-time PCR technique. In 18 (2.5%) patient analysis were unsuccessful due to insufficient material.

**Results:** From all successfully examined patients (n = 709, men = 501, 71%, women = 208, 29%), 65 (9.2%) were positive for TKI activating mutations in EGFR gene: men = 25 (5% of men); women = 40 (19% of women), p < 0.001. From 318 patients with squamous cell carcinosomas (SCC) 21 were positive (6.6%), p < 0.001. From 347 adenocarcinomas 43 were positive − 12.4%, p < 0.001. TKI activating mutations are more frequent in adenocarcinomas than in SCC (p = 0.009). From the rest 44 NSCLC one adenosquamous carcinoma (2.3%) was positive. From 108 never smokers 24 were positive (22.2%), from 9 past smokers one was positive (11.1%), from 215 ex-smokers 21 were positive (9.8%), from 321 active smokers 15 were positive (4.7%) p < 0.001. From patients with TKI activating mutations 37 had deletions in 19th exon (56.9%), 26 patients had L858R point mutation in 21st exon (40%), 1 patient − had L861Q point mutation in 21st exon (1.5%) and 1 patient had G719X point mutation in 18th exon (1.5%).

**Conclusion:** Our study confirmed that EGFR mutations are more frequent in adenocarcinomas, in women and in never smokers. We found that 6.6% of our patients with SCC have activating mutations in EGFR which is two times higher than in other studies (p < 0.001).

**[1147] Linear Combination Test for Gene Set Analysis of a Continuous Phenotype**

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**Background:** Gene-set analysis (GSA) tests association of sets of genes with a phenotype in DNA microarray studies. Many GSA methods have been proposed, especially for a binary phenotype. Most of these methods have been proposed, especially for a binary phenotype. Recent efforts have been made to address an important characteristic of microarray data, namely, the incorporation of correlations of gene expressions measurements within sets, into the test statistic. These methods have been proven superior in terms of power, while maintaining computational efficiency. While this task has been successfully accomplished in the case of binary and categorical phenotypes, there is still need for GSA methods incorporating the covariance matrix estimator for a continuous phenotype and, more generally for a continuous phenotype. Methods: We extend a GSA approach called Linear Combination Test (LCT), incorporating the covariance matrix estimator of gene expressions into the test statistic, from a binary to a continuous phenotype. Simulation studies and a
real microarray example are used to compare the proposed LCT, a modification of LCT, and two existing GSA methods for continuous phenotypic data.

Results: Our simulation studies show a good control of Type I errors by the four methods. LCT and its modification have a better control of Type II errors, compared to the other two existing GSA methods. Our simulation results are confirmed in an analysis testing 1,892 pathways and gene sets from the C2 catalog of the Molecular Signature Data Base (MsigDB), for association with tumor size measured in 250 patients from the Swedish Watchful Waiting cohort study on prostate cancer. A total of 6,144 gene expressions measurements were available for testing.

Conclusion: LCT is proven be both powerful and computationally attractive, due to incorporating the covariance matrix estimator via an orthogonal transformation.


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[1142] Comparative MicroRNA Expression Profiles in Human Cervical Cancer

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Introduction: Cervical cancer is the second leading cause of death among female cancer patients in the world. Clinical manifestation and progression are very diverse and not easily predictable.

Material and Method: Our aim was to identify differentially expressed microRNAs in cervical cancer cases from the Southwestern Transdanubian Region of Hungary in concordance with HPV-positivity, histological and clinical grading. After HPV-genotyping we analyzed the expression levels of 8 different microRNAs (miR-21, miR-27a, miR-34a, miR-146a, miR-155, miR-196a, miR-203, miR-221) in formalin-fixed-paraffine-embedded (FFPE) primary human cervical cancer samples. Expression profiles were evaluated and statistically analyzed in conjunction to HPV-status, histological and clinical grading.

Results and Discussion: The rate of high-risk (HR) HPV-positivity in squamous (SCC) and adenocarcinoma (ACC) of the cervix was 76% and 68.18% respectively. Out of the HR-HPV types HPV-16 and 18 were principally registered, with an overall prevalence for the two combined of 78.95% in SCC and 86.67% in ACC. HPV-16 was more frequently identified in SCC, than in ACC, and the difference was statistically significant (p = 0.041). The overall expression profiles based on the chosen 8 microRNAs were distinctive of the histological characteristics. The magnitude of expression levels of all miRNAs were higher in SCC, than in ACC, but the difference only reached the level of significance in the case of miR-21 (p = 0.038), miR-34a (p = 0.000) and miR-203 (p = 0.023). Further statistical analysis proved, that in SCC the level of miR-34a and miR-196a show significant correlation (p = 0.008 and p = 0.001 respectively) with clinical grading, while the association between HR-HPV-positivity and miR-155 expression levels was also significant (p = 0.048). In ACC none of the studied miRNAs showed significant associations with HR-HPV-positivity, but increased expression of miR-21 was significantly associated with FIGO stage of ACC.

Conclusion: Molecular characterisation of malignant dysfunctions will lead to a better understanding of mechanisms underlying the development of cervical cancer. The alterations we observed in miRNA expressions can be candidate gene targets and might even serve as possible predictive biomarkers in the field of prevention and therapeutic decision support in response to the urgent need for an earlier diagnosis, a more precise prognosis and a successful, personalized therapy.

[1149] Prognostic Factors and Prognostic Score of Relapsed Hodgkin’s Lymphoma

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Introduction: Depending on a stage and prognostic factors (PF), up to 95% patients with Hodgkin’s Lymphoma (HL) at the first presentation reach complete remission after standard treatment. Depending on their initial treatment, those patients relapsing have different treatment options, we identified PS for prognostic score (PS) which will help to optimize therapy.

Materials and Methods: From 428 patients II/IV stages with poor prognosis treated in N.N. Petrov Research Institute of Oncology between 1990 and 2006, 63 patients (15%) with early (n = 19) or late (n = 44) relapsed HL were identified. PS was calculated with modified clinical staging (CT) and twenty one (33%) after chemotherapy (CT). At relapse, characteristics of these 63 patients (median age, 34 years; range, 20 to 74 years) were stage III/IV, 67%; B symptoms, 35%; serum albumin <40 g/l, 21%; and Karnovsky performance score, less than 80 in 32%. At first relapse, salvage treatment was CMT in 13%, CT in 47%.

Results and Discussion: Median follow-up after relapse 106 months (range 24–237), 25-year overall survival (OS) for patients relapsing was 84%. The Karnovsky performance score, less than 80 (p = 0.0001), presence more than 1 extranodal localizations (p = 0.0004), III/IV stages at relapses (p = 0.001), B symptoms (p = 0.023), involvement of 5 and more areas of lymph nodes (p = 0.027), serum albumin <40 g/l (p = 0.037), new nodal involvement (p = 0.041) are defined by the discriminant analysis as predictor factors of response of second-line treatment at patients with relapsed HL. In multivariable analysis, independent PF for OS were failure after second-line treatment, early relapse occurrence, serum albumin <40 g/l, conventional-dose salvage CT in comparison with CMT. 5-year, actuarial 10-year OS for patients with failure after second-line treatment and complete remission was 78%, 40% and 95% (p = 0.0001). Patients with early relapse had 5-year, actuarial 10-year OS of 68%, 24% compared with 94%, 72% for late relapse (p = 0.01). 5-year, actuarial 10-year OS for patients with serum albumin <40 g/l and serum albumin >40 g/l was 69%, 23% and 78%, 46% (p = 0.02). Patients with salvage treatment CMT had 5-year, actuarial 10-year OS 88%, 63% and with only CT – 76%, 31% (p = 0.02). 5-year, actuarial 10-year OS was 95% and 88% for patients with PS (p = 0.60), and 86% and 28% for patients with PS 3−4 (p = 0.0002).

Conclusion: Prognostic factors and prognostic score for HL patients at relapse are important for considering in the choice of optimum therapy for the purpose of improvement of long-term survival and success of planned treatment.

[1150] Altered Glycosylation of Serum Glycoproteins as Tumour Biomarkers – the Case of Prostate-specific Antigen in Prostate Cancer and the Case of Acute-phase Proteins in Pancreatic Adenocarcinoma

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Background: Altered glycosylation is one of the hallmarks of tumour cells and it is involved in every and each aspect of tumour progression. It affects cell surface carbohydrates and carbohydrates secreted glycoproteins, some of which may reach the bloodstream and be used as tumour markers. The search for new tumour markers that could help in the management of cancer is required, especially diagnostic markers for earlier tumour detection. Our objective has been to determine whether some specific serum proteins carry altered glycans in cancer patients that could be potential tumour markers.

Material and Methods: Using immunoaffinity columns for serum depletion or immunoprecipitation of specific serum proteins, combined with N-glycan sequencing and specific carbohydrate antigen detection with antibodies against sialyl-Lewis structures, we have characterized the glycosylation of several serum proteins in cohorts of cancer patients compared to control patients.

Results: We have determined glycosylation changes of Prostate Specific Antigen (PSA) glycans, related to sialylation and fucosylation, in prostate cancer compared to Benign Prostate Hyperplasia and seminal plasma from healthy controls. In pancreatic adenocarcinoma, we have focused on glycosylation changes of acute-phase proteins (APP), some of which were found to be associated to inflammation. However, an increase in core fucosylated structures in the APP, alpha-1-acid glycoprotein and haptoglobin and increased sialyl-Lewis x in other APP such as ceruloplasm in were described and could be regarded as tumour specific.

Conclusions: We have described the altered glycosylation of serum proteins in prostate and pancreatic cancer as potential tumour markers. These tumour associated glycans changes should be investigated in a larger cohort of patients as possible cancer diagnostic or monitoring tools.

[1151] SNP Rs3219090 in the DNA Repair PARP1 Gene Reinforces the Protective Role to Melanoma Susceptibility in Spanish Population

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Background: Currently, vitamin D has been recognized as a prohormone with multiple functions to maintain optimal health. Vitamin D3 (cholecalciferol) is synthesized in skin exposed to sunlight. Cholecalciferol is converted in the liver in calcidiol, the most abundant in serum, by vitamin D 25-hydroxylase,
encoded by the CYP2R1 gene. Vitamin D sufficiency, along with controlled solar exposure, has been linked to lower risk of many cancers, including melanoma. The role of vitamin D on this vitamin dependence gene, CYP2R1, might modulate vitamin D levels as well as melanoma risk. Furthermore, we analyze several polymorphisms within DNA repair (PARP1), cell cycle regulation (CCND1, ATM), apoptosis (CASP8), immune response (MIZ) and vitamin D (GC, NADSYN1) pathway genes, with relevant results in recent melanoma GWAS (Genome wide association study). GWAS has arisen as a major approach to find candidate genes associated with diseases, however, results may be replicated in independent population such Spanish one.

**Material and Methods:** We present a case-control study in Spanish population including 521 melanoma patients and 339 control subjects. Six CYP2R1 SNPs with MAF > 0.05 were selected using HapMap database; seven relevant candidate genes were selected from previous GWAS on melanoma results. A total of 13 SNPs were analyzed by Kaspar technology. Results and Discussion: We found significant association between melanoma susceptibility and SNP rs3219909, located within PARP1 intron 13 (p-value 0.033). We also found marginal association with ATM SNP (p-value 0.15), which we are currently confirming in a large independent set. Conclusions: We offer a validation of previous results obtained on recent GWAS on melanoma patients; specifically we confirm the melanoma susceptibility protective role of PARP1, involved in DNA damage repair. We also show results that contribute to strengthen ATM as a candidate gene on melanoma susceptibility.

**1132 CYP2E1 and NQO1 Genotypes in Bladder Cancer − a Lebanese Case-Control Study**

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**Background:** Urinary bladder cancer is still the most prevalent type of cancer among Lebanese males. Exposure to carcinogenic, particularly arylamines and heterocyclic amines, has been shown to be directly associated with higher bladder cancer risk. Cytochrome P450 CYP2E1 and NADPH:quinone oxidoreductase1 (NQO1) are drug-metabolizing enzymes known to mediate metabolism of these carcinogenic. The main objective of the present study is to investigate a potential association between CYP2E1 and NQO1 genotypes and bladder cancer risk in a group of Lebanese males.

**Materials and Methods:** 54 cases and 105 hospital controls were randomly selected for the study from two major medical centers in the city of Beirut. Polymerase Chain Reaction-Restriction-Fragment-Length-Polymerism (PCR-RFLP) was performed on extracted DNA from peripheral blood samples to determine the CYP2E1 and NQO1 genotypes, and an interview-based questionnaire was completed to assess suspected environmental and occupational risk factors. Associations between bladder cancer and putative risk factors were measured using adjusted odds ratios (ORs) and their 95% confidence intervals (CI). Relative risk was derived using univariate, bivariate and multivariate logistic regression analysis.

**Results:** Data analysis highlights the CYP2E1-c2 allele as a potential risk factor for bladder cancer. Homozygous and heterozygous carriers of the CYP2E1-c2 allele had a 6.8 times higher bladder cancer risk. No significant differences in frequency distribution of the NQO1 genotypes were found in cases versus controls. Results also demonstrated that smoking, exposure to occupational fumes, and prostate-related symptoms, are risk factors for bladder cancer. Moreover, the risk of bladder cancer significantly increased in CYP2E1-c2 carriers not carrying the previously reported N-acetyltransferase 1 NAT1*T4A allele compared to carriers of both alleles.

**Conclusions:** Our study provides evidence that CYP2E1 genotype may play an important role in the development of bladder cancer among Lebanese men, and suggests a potential gene-gene interaction. Further studies with larger samples must be conducted to confirm these findings.

**1133 Colorectal Cancer Risk – the Influence of Selenoprotein Gene Variants and Blood Selenium Status**

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**Background:** Nutritional status evaluated by anthropometric and bioelectrical impedance in children and adolescents is a major concern for health professionals and caregivers. Studies show the impact of nutritional status on quality of life and morbidity, although there are few studies using bioelectrical impedance in this population. The aim of this study was to compare the nutritional status obtained by anthropometric and bioelectrical impedance in children and adolescents submitted to chemotherapy.

**Material and Methods:** We included patients admitted to the Hospital de Clínicas of Porto Alegre, aged between 4 and 18 years, who had attended at least one cycle of chemotherapy. We collected data on weight, height, arm circumference, triceps skinfold thickness and arm muscle area. Bioelectrical impedance was performed using the RJL Systems RJL-1300 Impedance Analyzer.

**Results and Discussion:** A total of 19 patients were evaluated. According to the values of z-score of body mass index for age, 10.5% of patients were considered malnourished, 52.6% as normal weight and 36.8% as overweight.

**1155 Comparison of Nutritional Status Obtained by Anthropometry and Bioelectrical Impedance in Children and Adolescents Submitted to Chemotherapy in a Hospital in Southern Brazil**

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**Background:** Studies show the impact of nutritional status on quality of life and prognostic in pediatric cancer patients. Body composition may affect mortality and morbidity, although there are few studies using bioelectrical impedance in this population. The aim of this study was to compare the nutritional status obtained by anthropometric and bioelectrical impedance in children and adolescents submitted to chemotherapy.

**Material and Methods:** We included patients admitted to the Hospital de Clínicas de Porto Alegre, aged between 4 and 18 years, who had attended at least one cycle of chemotherapy. We collected data on weight, height, arm circumference, triceps skinfold thickness and arm muscle area. Bioelectrical impedance was performed using the RJL Systems RJL-1300 Impedance Analyzer.
The percentile values of triceps skinfold and arm muscle area correlated with the nutritional status by body mass index for age, \( r = 0.81 \) and 0.67, respectively. The percentile of triceps skinfold thickness positively correlated with the percentage of fat mass \( (r = 0.74) \) and negatively with the percentage of lean body mass \( (r = 0.76) \). As previously described, we found a high percent correlation (\( \geq 0.85 \)) in these patients. Ours results also show a strong correlation between different parameters used for nutritional assessment.

**Conclusions:** Anthropometric parameters showed good correlation between them and with values obtained by bioelectrical impedance. Bioelectrical impedance is an accurate method for nutritional assessment and measurement of body compartments in pediatric patients with malignancies.

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**[115A]** A High Prevalence of PALB2 Mutations Among Familial Breast Cancer Patients in the Czech Republic

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**Background:** A positive family history is an important risk factor for the development of breast cancer. Along with the BRCA1 and BRCA2 genes, mutations in the two major BC susceptibility genes, BRCA1 and BRCA2, account for only 20–25% of all BC cases. More than 70% of hereditary cancers may thus be associated with mutations affecting other predisposing genes. The PALB2 protein (for Partner And Localizer of BRCA2) is a DNA damage sensor and repair regulator. PALB2 mutations affect the repair of double-strand breaks (DSBs) in the genome, and are inherited in autosomal-dominant pedigrees. Several studies have demonstrated a high frequency of PALB2 mutations in breast and ovarian cancer patients.

**Material and Methods:** We screened a total of 332 high-risk BC patients for PALB2 mutations using Sanger sequencing of the whole coding region of the gene. Analyzed group included 270 familial cases and 62 non-familial cases (bilateral BC, male BC, breast and ovarian tumor duplicity). Identified mutations were analyzed in a group of 1272 control samples. MLPA was used for the detection of large genomic rearrangements.

**Results:** In total we found 10 different pathogenic mutations in 13 patients, whereas one mutation was observed in controls (\( P < 0.001 \)). Twelve mutations were detected in 187 familial cases (6.4%) with a family history positive for BC only, whereas no mutations were detected in 83 families with ovarian cancer. One mutation was identified in 19 male BC cases (5.3%). Six frameshift and 3 nonsense mutations were identified by sequencing; MLPA technique revealed a large deletion including exons 9–10. Seven of the ten identified mutations were novel. A 4 base-pair deletion (c.172_175delTTGG) was found in four subjects and was the only recurrent mutation identified in the PALB2 gene. Analysis of this mutation was extended to a group of 704 unselected BC patients where it was identified in 2 cases (0.28%). Four of six carriers of this mutation shared the same haplotype suggesting a common origin of this mutation.

**Conclusion:** Analysis of the PALB2 gene confirmed its role in BC susceptibility in the Czech population. High frequency of PALB2 mutations identified in high-risk BC families strongly suggests the relevance of clinical testing of this gene.

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**[1157]** Association of LINE-1 Methylation With Risk of Bladder Cancer in the Spanish Population

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**Background:** Global DNA hypomethylation has been suggested to increase the risk of several cancers, among them bladder cancer (UCB). The objectives of this study were to assess the association between global DNA methylation, measured as long interspersed nuclear element 1 (LINE-1) methylation level and UCB risk, and (2) to identify effect modifiers, determined by environmental factors and genetic variants mainly from the one-carbon metabolism pathway.

**Material and Methods:** Cases and controls were recruited to the Spanish Bladder Cancer Study from five regions in Spain between 1998–2001 and matched for age, gender and area of residence. Sodium bisulfite-treated leukocyte DNA from 952 cases and 892 controls was used to measure LINE-1 methylation level at four randomly selected CpG sites by pyrosequencing. Multivariate logistic regression models, adjusted for age, gender, area, and smoking, were used to estimate the odds ratios (OR) and 95% confidence intervals (CI) between LINE-1 methylation level and UCB risk.

**Results:** The risk of UCB followed a U-shaped pattern association with LINE-1 methylation. Compared to subjects in the middle tertile, the adjusted OR for subjects in the lowest and the highest tertiles were 1.26 (95% CI 0.99–1.60, \( p = 0.06 \)) and 1.33 (95% CI 1.05–1.69, \( p = 0.02 \)), respectively. This association was significantly modified by five single nucleotide polymorphisms (LD, \( r^2 > 0.83 \)) located in the phosphodiesteranline N-methyltransferase (PMT) gene, homozygous individuals for the major allele showing the highest risk. Corrected interaction \( p \)-values: rs2124344 (\( p = 0.01 \)), rs4646340 (\( p = 0.03 \)), rs4646341 (\( p = 0.04 \)), rs4646350 (\( p = 0.03 \)), rs7215833 (\( p = 0.02 \)). Smoking exposure to trace elements, and diet did not modify the effect of LINE-1 methylation on UCB risk.

**Conclusions:** This is the first large scale study showing that both low and high levels of global DNA methylation levels are associated with risk of UCB. Moreover, variants in PMT seemed to markedly modify this risk. These results may provide additional insights into the mechanisms through which changes in DNA methylation associate with UCB development. These findings deserve further exploration in independent populations.

**[1155]** Candidate Gene Association Study in Childhood Acute Lymphoblastic Leukemia

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Childhood acute lymphoblastic leukemia (ALL) is the most common pediatric cancer and its etiology remains poorly understood. It has long been known that ALL have a genetic background investigated through association studies based on candidate genes that involved in transcriptional regulation and differentiation of lymphoid progenitors. ARID5B (AT-rich interactive domain 5B) is a novel susceptibility factor for childhood ALL and plays a crucial role in the regulation of embryonic development, cell growth and differentiation. Ikaros proteins (coded by IKZF1-Ikaros zinc finger 1 gene) are master regulators of lymphocyte development, and differentiation. Our study provides more detailed analysis to identify the causal variants of ARID5B and IKZF1 susceptibility to childhood ALL.

In 543 children with ALL and 529 controls we genotyped 62 single nucleotide polymorphisms (SNPs) in 20 candidate genes and investigated whether the presence of these polymorphisms was associated with the disease. The genomic DNA was obtained retrospectively from whole, peripheral blood in remission. Genotyping of the selected SNPs was carried out by Sequenom iPLEX Gold MassARRAY technology (McGill University and Géneome Québec Innovation Centre, Canada). The results were evaluated with traditional frequentist-based methods and a new one, called Bayesian network based Bayesian multilevel analysis of relevance (BN-BMLA). We found that six polymorphisms in two genes influenced the risk of ALL significantly: rs11978267, rs4132601, rs6964969 in IKZF1, and rs10821936, rs4506592, rs7080442 in ADRI5B. The most relevant SNPs were: rs10821936 (\( p = 7.31 \times 10^{-5} \); OR=1.430; 1.198–1.706) and rs6964969 (\( p = 1.67 \times 10^{-5} \); OR=1.497; 1.246–1.800) in the whole population and even in the subgroups. With BN-BMLA we also computed the a posteriori probability of the different association types with respect to ALL susceptibility in all sample groups. According to the BN-BMLA method the major SNPs and genes (i.e. with high posterior for strong relevance) were rs10821936 in ARID5B (Posteriori = 0.76) and rs4132601 (Posteriori = 0.97) in IKZF1. In our presentation we also show some examples how the different BN BMLA results can be interpreted. Our results contribute to the understanding of genetic basis of ALL development. Better elucidation of the mechanisms through which ARID5B and IKZF1 variants are involved in childhood ALL could be of great diagnostic value, and help to improve risk directed therapy and disease outcome.

**[1159]** Diversity of HPV Genotypes in Cervical Tumors From the Rio de Janeiro, Brazil

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**Introduction:** Cervical cancer is the second most common cancer among women worldwide, with approximately 500,000 new cases/year and 230,000 deaths. The incidence is about two times higher in less developed countries when compared to developed countries. HPV16 and HPV18 are the most frequently viral types associated to cervical cancer and high-grade cervical neo-
plasia. This prevalence lead to the development of two commercial vaccines against these two types. In Brazil, are expected ca. 17,000 new cases in 2012, and there are few studies characterizing the HPV types present in cervical tumors. Given the relevance of cervical cancer, its association with different types of HPV, the development of vaccines, and the limited number of studies in Brazil, our goal is to estimate the prevalence of different HPV types in cervical tumors samples from women referred to the Instituto Nacional de Cancer, Rio de Janeiro, Brazil, and its association with epidemiological data.

Material and Method: The population of the study was composed of 72 women with de novo cancer, enrolled between July 2011 and October 2011. Briefly, the DNA was extracted from biopsies of tumor tissue with the QIamp DNA Mini Kit (QIAGEN) and the presence of HPV DNA was detected using the previously reported PGMY-PCR method, the amplicons were directly sequenced, and HPV types were identified using Blast (http://blast.ncbi.nlm.nih.gov).

Results and Discussion: The average age of the biopsied women was 48.8 years old, being 30.6% white, 19.4% black, and 50% brown; the average of years of formal education was 5.9 years for white women, 5.7 for black women, and 5.1 for brown women; the average familial income was US$ 699.00 (median= US$ 840.00). Squamous cell carcinoma were found in 69% of women and adenocarcinoma in 15%. Presence of HPV was found in tumors from 71 women, being: 47 samples with HPV16 (65.3%), 5 with HPV58 (6.9%), 5 with HPV18 (6.9%), 2 with HPV33 (2.8%), 2 with HPV45 (2.8%), 2 with HPV59 (2.8%) and one sample with each of types HPV06, HPV39, HPV52 and HPV68. In three samples was not possible to identify the HPV type present, and in one sample the viral DNA was not detected.

Conclusion: In conclusion, we found that HPV16 was the most prevalent and, interestingly, HPV58 and HPV18 were the next most prevalent types, with identical frequency. HPV58 and HPV18 were associated with poor prognosis, being possible biomarkers of disease evolution. Supported by: FAPERJ (E-26/102.235/2009); CNPq (478564/2011-2). INCA.

[1165] Prognostic Impact of Chromosomal Abnormalities and N-ras Mutations In Patients With Primary Myelodysplastic Syndrome Treated With Allogeneic Hematopoietic Stem Cell Transplantation

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Background: Primary MDS is a heterogeneous group of clonal disorders of hematopoietic stem cells. The only potential curative treatment for MDS patients is allogeneic HSCT. However, since transplantation is a procedure of high toxicity, mortality, and morbidity, the selection and timing to prescribe this treatment to MDS patients is a difficult task. The present study focused on the analysis of different prognostic factors in patients with MDS treated with allogeneic HSCT, especially the impact of cytogenetics.

Material and Methods: The analysis covers the period between 1991 and 2010, 84 adult patients (53 RA, 18 RAEB and 13 RAEB-t) and 40 pediatric patients (28 CR, 9 RAEB and 3 RAEB-t) were evaluated. Cytogenetic analysis was performed by GTG banding and FISH. N-ras point mutation analysis was performed by PCR and the samples were sequenced.

Results: Clinical follow-up after allogeneic HSCT showed that of the 124 patients, 13 (10%) had graft failure. Relapse was observed in 29 patients, 10 children (34.5%) and 19 adults (65.5%), and the presence of GVHD was found in 62 patients (50%). Relapse had the greatest impact on patient survival and was strongly associated with the presence of abnormal karyotypes and high percentage of blasts. The IPSS was found to be a good scale for patients treated with transplantation, but the intermediate cytogenetic risk group needs to be reviewed, for example, +8 and del (11)(q23) in our study were associated with the relapse of disease. A small percentage of patients (17%) showed relapse with normal karyotypes. This led us to investigate the presence of mutations in N-ras gene. We verified the mutation in 25 (21%) of the 117 patients studied pre-allogeneic HSCT with higher N-ras frequency of mutations in adult patients. N-ras mutations were associated with relapse of the disease in adult patients with normal karyotypes. Patients with N-ras mutations and abnormal karyotypes showed recurrence in a shorter time period than those with only abnormal karyotypes, so did the patients with N-ras mutations and normal karyotypes.

Conclusions: Our results suggest that patients with primary MDS should be indicated for allogeneic HSCT in the early stages of the disease and that the cytogenetic plays an important role in the selection, indication, and monitoring of patients with MDS treated with allogeneic HSCT.

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Introduction: Night work has been associated with an excess risk of breast cancer in nurses working at night. The hypothesis is that night work and exposure to artificial light at night may disrupt the circadian rhythm. Polymorphisms in circadian genes, in combination with night work, may contribute to individual differences in susceptibility to breast cancer. Up to date, only few polymorphisms in circadian genes have been investigated in relation to susceptibility to breast cancer, in combination with night work. In this study, we have performed a comprehensive analysis of 61 single nucleotide polymorphisms (SNPs) in 17 circadian genes in 1182 Norwegian nurses and have analyzed the data in relation to occupational exposure to night work.

Materials and Methods: The case-control study was nested within a cohort of 49,402 Norwegian nurses, aged 35–74 years. The cases (n = 563) were frequency matched with controls (n = 619). The schedules of night work included only day shifts, only night shifts, or both day and night shifts. The DNA was extracted from saliva and genotyped for a total of 61 SNPs selected in 17 circadian genes. The odds ratios (OR) were calculated using unconditional logistic regression.

Results: The analysis, without regard to night work, showed that only 3 SNPs were associated with breast cancer risk: carriers of variant TT genotypes of SNP rs2278749 in the ARVT1/1BLM/1 gene had reduced risk of breast cancer.
Thyroid Peroxidase, Galectin-3, Cytokeratin-19 and Hector TP53 Mutational Signature of Aristolochic Acid in Carcinomas

Department of Nephrology and Arterial Hypertension, Zagreb, Croatia, Department of Endocrinology and Radioimmunology, Belgrade, Serbia, shifts (4 consecutive nights) only 1 SNP (rs10838524) in the genes CLOCK, PER1 and RORA were found to be significantly associated with risk whereas in subjects who had worked 3 consecutive night shifts 6 SNPs in the genes ARNTL/BMAL1, ARNTL/BMAL2, NPAS2, PER3, ROR-6 and MTNRA1 were found to be associated with risk. Interestingly, in subjects with highest number of consecutive night shifts (4 consecutive nights) only 1 SNP (rs10838524) in the CRY2 gene was associated with risk.

Conclusion: Several SNPs in various circadian genes in combination with night work were found to modify risk of breast cancer.

Thyroid Peroxidase, Galectin-3, Cytokeratin-19 and Hector Battifora Mesothelial Antigen-1 Expression in Papillary Thyroid Carcinoma and Their Relation to Clinicopathological Features

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Introduction: Thyroid peroxidase (TPO), galectin-3 (gal-3), cytokeratin-19 (CK-19) and Battifora mesothelial antigen-1 (HBME-1) were reported as useful markers of thyroid malignancy. To assess their value as prognostic markers of papillary thyroid carcinoma (PTC), the most frequent carcinoma of thyroid gland, we have evaluated the expression of these proteins in PTC samples and obtained results were correlated with unfavorable clinicopathological features of the patients.

Material and Methods: A total of 147 archival thyroid tissue samples of papillary thyroid carcinoma were analyzed by immunohistochecmistry. The absence of TPO (<5% stained thyrocytes) and high expression of gal-3, CK-19 and HBME-1 (>50% stained thyrocytes) were correlated with the gender and age of patients, tumor size, multifocality and extrathyroidal invasion, lymph node metastasis, depth of tumor invasion (pT status) and tumor-node-metastasis classification (pTNM stage). The results were evaluated by univariate (correlation, χ2, Student’s t, ANOVA, Kruskal-Wallis’s test) and multivariate set of tests (Binary logistic regression and Discriminate analysis).

Results: Absence of TPO, as well as high expression of CK-19, were associated with the age of patients (p < 0.05), extrathyroidal invasion of the tumor (p < 0.01) and pT status (p < 0.05). In addition, absence of TPO could be a predictor of higher pTNM stage (p < 0.01) while high expression of CK-19 was correlated with tumor multifocality (p < 0.05). Furthermore, high levels of expression of HBME-1 and gal-3 were significantly associated with lymph node metastasis. Therefore, high expression of gal-3, CK-19 and HBME-1 indicate more aggressive biological behavior of PTC. On the contrary, papillary carcinomas that displayed greater TPO expression were predominantly less aggressive as indicated by the pTNM ranking.

Conclusion: Assessing immunohistochemical staining for TPO, gal-3, CK-19 and HBME-1 could allow identification of papillary thyroid carcinoma patients who are at higher risk for a worse prognosis and should be controlled more frequently.

TP53 Mutational Signature of Aristolochic Acid in Carcinomas of the Upper Urinary Tract

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Background: Retinoblastoma (RB) is a childhood tumor with an annual incidence of 12 to 27 cases per million in Brazil. RB1 tumor suppressor gene, the main causal gene, is located in 13q14.2 and presents 27 exons. Retinoblastoma protein acts in cell cycle arrest by binding E2F transcription factors, which prevents S phase initiation. Carriers of germline or embryonic pathogenic constitutional mutations tend to develop bilateral tumor, and 15% of carriers of unilateral tumor also reveal constitutional mutations. If a patient does not show germline mutation, the risk of tumor recurrence in subsequent siblings and generations is smaller. For patients who display germline mutations, however, it is necessary to investigate their parents and siblings, as approximately 10% of mutation carriers are asymptomatic. The study aims to investigate the presence of constitutional RB1 abnormalities using a complementary method in samples from RB patients seen at our clinic, in order to gather relevant data for genetic counseling of patients and their families.

Materials and Methods: DNA is obtained from peripheral blood leukocytes by ‘salting out’ protocol. The 27 RB1 exons are amplified by PCR, purified with a commercial kit (QiAquick PCR Purification Kit − QiAGEN) and sequenced. If no mutations are found, a commercial kit for MLPA (Multiplex Ligation-dependent Probe Amplification) reaction is used for detection of deletions/duplications in RB1 gene/13q region.

Results and Discussion: To date, 30 RB DNA samples have been analyzed. Preliminary analysis revealed the presence of total and partial deletions in some patients, including entire RB1 deletion and flanking genes. In some cases, a single or two consecutive RB1 exons were deleted; in one case, promoter region and exon 1 were deleted and, in another case, RB1 gene was partially deleted. Patients investigated so far did not show any mutation.

Conclusions: These results signaled to the importance of evaluating the proband and his/her relatives with a complementary method, as many patients have a constitutional mutation non-detectable by sequencing, which could be shared by other at-risk family members. Results also showed the efficiency of searching mutations by ligation-dependent probes, which has proved to be an effective method to identify copy alterations in RB1 locus.
**Polymorphisms of the EGFR Gene and Association With Reduced Metastasis in Non-small Cell Lung Cancer**

Other and is associated with favorable prognosis in colorectal carcinoma and with functions in ligand binding, tyrosine kinase activation and growth stimulation, and development and growth by regulating the differentiation of cells and tissues. EGFR is frequently overexpressed in many tumors, including breast cancer, and contributes to unrestricted proliferation. The polymorphism R497K (rs11543648), located in the exon 13 of the EGFR gene, leads to attenuated functions in ligand binding, tyrosine kinase activation and growth stimulation, and is associated with favorable prognosis in colorectal carcinoma and with reduced metastasis in non-small cell lung cancer. Other EGFR polymorphisms are (CA)n dinucleotide repeat sequence in intron 1 (rs72554021). Shorter sequences, i.e. (CA)n <16 dinucleotide repeats are associated with increased EGFR transcriptional activity in breast tumors. The aim of the present study was to investigate the association between EGFR polymorphisms and histopathological variables with prognostic value in breast cancer.

**Material and Method:** The study protocol was approved by the Ethics Committee of the Brazilian National Cancer Institute (NCT02806) and consisted of a prospective cohort study in women (age >18 years old) with a confirmed diagnosis of unilateral non-metastatic breast cancer. Genomic DNA was extracted from blood samples and the polymorphism R497K was identified by PCR-RFLP in 472 patients, whereas the fragment length of the intron 1 was determined by capillary electrophoresis in 426 patients. The histopathological profile was determined after tumor resection. The association between EGFR genotypes and histopathological features was evaluated by the Chi-square test. Odds ratios (OR) and their 95% confidence intervals (95% CI). Results and Discussion: The frequency of the Lys allele was 0.218 (95% CI, 0.192–0.245), and the genotypic distribution was in Hardy-Weinberg equilibrium (p = 0.249). Eleven different (CA)n alleles were found, ranging from 14 to 24 repeats, forming thirty seven different genotypes. The major frequent allele was (CA)n16 repeats (0.425; 95% CI, 0.398–0.460). Patients with at least one Lys allele showed a lower chance of presenting lymph node status N2 or N3 (OR = 0.419; 95% CI, 0.231–0.759, p = 0.008), which contributed for lower stages in TNM status (OR = 0.269–0.851, p = 0.011), and lower estimated risk of recurrence (OR = 0.470; 95% CI, 0.278–0.794, p = 0.004), than those who were Arg/Arg. Patients with two long alleles, i.e. (CA)n >16 repeats, showed a higher chance of being positive for progesterone receptor (OR = 1.738; 95% CI, 1.040–2.904, p = 0.021). Patients Arg/Lys with two long (CA)n alleles and patients Lys/Lys with at least one long (CA)n allele showed a lower estimated risk of recurrence (OR = 0.169; 95% CI, 0.052–0.554, p = 0.001).

**Conclusion:** The combined data of R497K and (CA)n repeat polymorphism suggests that the presence of the variant forms of EGFR contribute for better prognosis in breast cancer.

**Role of Xenobiotic Metabolizing Gene Variants in Bladder Cancer Susceptibility**

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Introduction: Occupational exposure to carcinogens and cigarette smoking are among the most significant risk factors for bladder cancer. These carcinogens are metabolized by phase-I and phase-II enzymes of the xenobiotic metabolism. It is accepted that the interaction between genetic factors and environment accounts for different levels of susceptibilities to the development of bladder cancer. The frequent coding for CYP superfamily of enzymes, AKR1C3 and NQO1 have been assessed in a case-control study using PCR-RFLP.

Results: We have firstly studied the CYP1A1, CYP1B1, CYP2D6 and CYP2E1 SNPs in 62 bladder cancer patients and 73 healthy subjects. According to the results, CYP1B1 432Val allele was found to increase the risk of bladder cancer (OR = 3.08; 95% CI = 1.20–7.874; adjusted for age and sex). The effect of AKR1C3 rs12529 polymorphism was investigated in 101 bladder cancer cases and 101 healthy controls. We have identified that men (OR = 0.932; 95% CI = 4.308–19.938) and cigarette smokers (OR = 4.89; 95% CI = 2.617–9.863) are under high risk for developing bladder cancer and that homozgyote variant (GG) homozygote variant can inhibit the procarcinogenic effect (OR = 0.26; 95% CI = 0.101–0.643) compared with the wild type homozgyote (CC). The protective effect of the GG genotype becomes more apparent when the odds ratio is adjusted for cigarette smoking and sex (OR = 0.243; 95% CI = 0.743–0.85–0.6). These results indicate a strong relationship between AKR1C3 rs12529 polymorphism and bladder cancer susceptibility. We have also examined the NQO1 C697T polymorphism in 174 bladder cancer patients and 152 control patients; however this polymorphism was not found to be associated with bladder cancer risk. On the other hand, men appeared to have 5.58 fold more risk for bladder cancer compared to women, and cigarette smokers carry 3.85 fold higher risk than non-smokers (p = 0.05).

**Conclusion:** CYP1B1 rs1056836 and AKR1C3 rs12529 were found to be associated with bladder cancer risk. Cigarette smoking with CYP1B1 432Val allele were found to have 3 fold increased risk of bladder tumor formation. On the other hand, AKR1C3 rs12529 GG genotype was found to confer protection against bladder cancer.

**Gender Differences in Global Survival Among Patients With Oral Squamous Cell Carcinoma**

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Squamous cell carcinoma (SCC) is the most common tumor in oral cavity and its profile is well recognized - elderly Caucasian men, with low social-economic situation diagnosed in advanced stages. In the last decades changes in women's lifestyle have contributed to the increasing number of cases in females. The aim of this study was to determine if socio-demographic, clinical and pathological prognostic factors can promote differences in global survival between men and women with OSCC.

The study population was surgically treated at the National Cancer Institute from 1999 to 2003 and comprised 480 patients. For statistical analyzes the chi-square or Fisher's exact test were used, as well as the Kaplan–Meier method for survival analysis and Cox regression model to verify the independence among variables. A p-value < 0.05 was considered statistically significant. The Statistical Package for Social Sciences (SPSS) was employed in the analysis. Patients were mostly male (72.5%) with a mean age of 56.3 years. Women represented 34% cases with a mean age of 62.7 years. Differences between genders were observed - women consumed less amount of alcohol than men, and women had a higher Body Mass Index (BMI). Widows were most common among females and males were mostly married. Men had tumors in more advanced pathological stage compared to women, which also presented smaller tumors. Better survival rates were observed in older males (p = 0.03), with a high BMI (p < 0.001), presenting well-differentiated tumors (p = 0.01) in initial clinical staging (p = 0.01), submitted to salvage surgical treatment (p = 0.03), and without cervical metastasis (p < 0.001). For males, it was found that married women (p = 0.02), with gingival tumors (p < 0.001), using a combination of different drinks (p = 0.03), submitted to salvage surgical treatment (p < 0.001) and without cervical metastasis (p = 0.01) showed better survival rates. Cox regression revealed that age (p = 0.01), treatment (p = 0.01) and histopathological grading of the specimen (p = 0.05) were independent prognostic factors for males; while type of beverage (p = 0.01) and treatment (p < 0.001) were independent prognostic factors for females.

These results showed that prognostic factors for oral squamous cell carcinoma are different between men and women and it's important to recognize them to propose social support as a part of the treatment plan.

**Genetic Modulation of Lung and Skin Tumorigenesis by the Mouse Lsktm1 Locus**

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Introduction: The non-inbred carcinogenesis-resistant (Car-R) mouse, derived from the crossing of 8 inbred lines, was phenotypically selected both skin and lung tumorigenesis, and the skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) in crosses between Car-R and SWRJ mice, which are susceptible to skin and lung cancer, showed that the Car-R genotype significantly reduced that age (>90% of animals) (p = 0.001), treatment (p = 0.01) and histopathological grading of the specimen (p = 0.05) were independent prognostic factors for males; while type of beverage (p = 0.01) and treatment (p < 0.001) were independent prognostic factors for females.

These results showed that prognostic factors for oral squamous cell carcinoma are different between men and women and it's important to recognize them to propose social support as a part of the treatment plan.
linkage analysis of the SWRJ × (SWRJ × Car-R) backcross population using high-density marker coverage provided by single nucleotide polymorphism (SNP) array. Gene expression analysis of non-tumor tissues from Car-R and SWRJ was performed; then, we tested the effects of candidate genes (lgfbp2 and lgfbp5) overexpression on clonogenicity of lung cancer lines. Finally, functional analysis of the lgfbp5 promoter alleles from SWRJ and BALB/cJ by luciferase reporter assay was performed.

Results and Discussion: We identified the lung and skin tumorigenesis modifier 1 (Lsktm1) locus on chromosome 1 linked to both skin (LOD score = 3.93) and lung (LOD score = 8.74) tumorigenesis. The Lsktm1 locus was confirmed in a (SWRJ × BALB/cJ)F2 intercross population, indicating that BALB/cJ carries the same cancer resistance alleles of Car-R mouse. Two genes, lgfbp5 and lgfbp2, residing in this locus and belonging to the insulin-like growth factor binding protein family, were expressed at significantly higher levels in normal lung tissue from cancer-resistant Car-R mice than in cancer-susceptible SWRJ mice, and overexpression of the recombinant genes in two lung cancer cell lines significantly inhibited clonogenicity (P < 0.0001). Functional analysis of the lgfbp5 promoter alleles from SWRJ and BALB/cJ by luciferase reporter assay showed differential expression between the strains (P = 0.0001), suggesting a possible involvement of lgfbp5 promoter variants in modulating gene expression. Conclusion: Collectively, our results demonstrate that skin and lung tumors are influenced by a single polymorphic genetic locus and identify lgfbp5 and lgfbp2 as candidate modifier genes of lung tumorigenesis.

DietCompLyf Study — A multi-centre UK Study on Breast Cancer — What Are the Dietary and Lifestyle Changes Following Diagnosis?

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Background: Evidence summarising the influences of dietary and lifestyle factors in prevention of breast cancer is now established, allowing a focus of research toward learning the independent and combined effects of features including alcohol, polysaturated fat intakes and exercise regimes on breast cancer outcomes. Method: The DietCompLyf study recruited 3,390 female breast cancer patients from 56 NHS centres across the UK. Blood and urine samples were collected alongside information on lifestyle, diet, psychological well-being and quality of life annually for 5 years. Describing the DietCompLyf cohort using tumour biology and treatment profiles, participants’ geographical location, age and ethnicity, the demographics of this cohort are compared with UK breast cancer rates. Additional features specific to the cohort including the socio-economic status, general health and alcohol consumption are also considered alongside changes made to the diet and lifestyle since diagnosis. Multivariable analysis (multiple linear regression) on the changes in BMI and its causes was carried out.

Results and Discussion: Recruitment was closed in September 2010 and the collection and validation of baseline data completed. 231 records were excluded for data violation. Provisional analyses of socio-demographic data on the final dataset (3,161 genes) and over 3,700 more in trans (2,275 genes). Though the expression of this large number of genes was modified by SNPs in their genomic region, our hypothesis has not been verified. The distribution of p-values was the same for the eQTL than for the remaining SNPs.

Conclusions: Though eQTLs modify the expression of the gene, this effect doesn’t seem to have a relevant impact in the likelihood of these SNPs being susceptibility loci. A more detailed analysis will be presented, studying specifically cancer genes and the distribution of eQTLs in cancer pathways.

Expression of LASS/CerS 4 and 5 in Colon and Endometrial Cancers

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Introduction: LASS/CerS genes belong to a family of six related genes (CerS1–6). CerS gene products have been shown to produce ceramide, hence their name CerS. Ceramide has been implicated in cancer and apoptosis. A proper regulation of the balance between cell growth and cell death is essential for cellular homeostasis. The precise roles of CerSes in different cancers is not fully understood, especially the role of CerS4 and CerS5 in colon and endometrial cancers. The broad aim of this study was to investigate the role of CerS4 and CerS5 in apoptosis and cancer.

Materials and Methods: Bioinformatics tools were used to determine the conservation of these genes in different organisms, and to determine their potential protein-interacting partners, to shed light on their possible roles in cancer. Apoptosis was induced in cultured colon and endometrial cancer cells with their non-cancerous counterparts using 5-FU and Anastrozole respectively. Fluorescence activated cell sorting (FACS) was used to analyse and quantify apoptosis in the untreated and treated cells. Total RNA was then extracted from both treated and untreated cells for cDNA synthesis. The synthesised cDNA was subjected to real quantitative real-time PCR using LASS4 and LASS5 specific primers. The results were normalised using β-actin.

Results and Discussion: LASS4 and LASS5 genes were shown to be conserved throughout evolution, in different organisms. CerS4 and CerS5 were found to potentially interact with other proteins that may be involved in cell cycle regulation. The cancerous cells showed a higher susceptibility to apoptosis inducing drugs in both colon and endometrial cancers. Using the ANOVA test, FACS analysis results were shown to be statistically significant. Using quantitative real-time PCR, LASS4 and 5 were shown to be up-regulated in both colon and endometrial cancer cell lines. However, the expression of CerS4 and 5 were down-regulation of both LASS 4 and LASS 5 in these cancers. Using the Turkey’s test, the results were shown to be statistically significant. These findings implicate the involvement of these genes in cancer and apoptosis.

Colorectal Cancer eQTLs as Susceptibility Loci Candidates

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Background: The identification of genetic susceptibility loci in colorectal cancer genome-Wide Association Studies (GWAS) has yielded so far a few hits, but most of the heritability remains to be explained. The identified loci have small effects and, generally, no idea of the carcinogenic mechanisms behind the loci have been proposed. GWAS analysis provide lists of candidate SNPs but only a few, generally the most significant, are tested in validation studies. Here we hypothesise that SNPs associated to gene expression in normal tissue (eQTLs) have a higher prior probability of being involved in the carcinogenic mechanism. If true, SNPs that are eQTLs should show more significant p-values than expected in a GWAS.

Material and Methods: Normal mucosa from 100 patients with colon cancer and 100 healthy donors that underwent careful endoscopy and colonic biopsies. LINKAGE arrays U219 for gene expression and Human Genome 6.0 for genetic variation. To reduce spurious associations, filters were applied beyond quality standards (missing values <10%). Expression probes with low variability (sd <0.26) or low expression (mean <2.4) were excluded. SNPs with HWE p <0.0001 or MAF <0.1 were also excluded. A total of 559,156 SNPs remained for analysis. QTLs were analyzed assuming a linear model on expression, adjusted for cancer status, age and sex. The significance level used for eQTL associations (SNPs within 1 MB of the target gene) was 4e-4, and for trans association 1e-7. Associations that showed heterogeneity between cases and controls were excluded. The R package MatrixEQTL was used for these analysis. GWAS data was available from the EPICOLON study, a case-control study that included 489 cases of colorectal cancer and 629 controls recruited in Spain. Affymetrix Human Genome 6.0 had also been used in this study. The trend test was used to summarize the association of each SNP with colorectal cancer.

Results: More than 23,000 SNP-gene associations were identified in cis (3,161 genes) and over 3,700 more in trans (2,275 genes). Though the expression of this large number of genes was modified by SNPs in their genomic region, our hypothesis has not been verified. The distribution of p-values was the same for the eQTL than for the remaining SNPs.

Conclusions: Though eQTLs modify the expression of the gene, this effect doesn’t seem to have a relevant impact in the likelihood of these SNPs being susceptibility loci. A more detailed analysis will be presented, studying specifically cancer genes and the distribution of eQTLs in cancer pathways.
Furthermore, exploiting two isoforms of the same gene could aid in dissecting and understanding the roles of these CellS transcripts at gene expression level.

**Conclusion:** Targeting this family of genes and understanding their precise individual roles, is a promising therapeutic tool to new anti-cancer drug discovery or improving the existing ones.

**[1174] Association of RBBP6 With Cervical Cancer Progression**

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**Introduction:** RBBP6 is a nuclear protein, previously implicated in the regulation of cell cycle and apoptosis. It is a multi-domain protein containing a Zinc finger, a RING finger, a Rb binding domain, a p53 binding domain and a novel N-terminal protein domain, DNN. Recently, RBBP6 has been implicated in lung cancer development while isofrom 3 deregulation has also been implicated in cancer development. The purpose of the study was to elucidate the possible role of RBBP6 in cervical cancer and apoptosis, to establish tissue distribution and expression levels of DNN at protein and mRNA levels in cervical cancer.

**Materials and Methods:** In situ hybridization and immunohistochemistry were used to localize RBBP6 mRNA and proteins in cervical cancers respectively. Real time was used to further determine the expression of RBBP6 mRNA. K67 and Bcl-2 were also localized to determine associations between these proteins and RBBP6 while the TUNEL assay was used to determine the apoptosis levels in this cancer.

**Results and Discussion:** In situ hybridization studies showed elevated levels of RBBP6 mRNA transcripts in cervical cancer as compared to the normal tissues. The transcripts were localized in the nuclei of invaded stroma, moderately differentiated islands of tumours, dysplastic epithelium and some infiltrating lymphocytes. Immunohistochemistry showed that RBBP6 proteins were highly expressed in the dysplastic epithelium, dysplastic endocervical glands, moderately and well differentiated islands of tumours and the invaded stroma. High apoptotic levels in the invaded stroma and moderately differentiated islands of tumours and this study significantly suggested association with RBBP6 localization. K67 and Bcl-2 expressions were found to be indirectly proportionally to RBBP6 expression. The up-regulated levels of RBBP6 in cervical cancers in contrast to normal tissues suggest RBBP6 to be pro-apoptotic, as there were elevated levels of apoptosis in the same sites where there were high levels of RBBP6 expression and Bcl-2 was down-regulated in the same sites. There was association between RBBP6 expression and apoptotic levels while this expression was indirectly proportional to K67 in human cervical cancers. Real Time PCR also confirmed the up-regulation in levels of RBBP6 in cervical cancer.

**Conclusion:** This study further suggests that the RBBP6 gene's involvement in human cancers and could be a target for both diagnostics and therapeutics.

**[1175] Genetic Polymorphisms of SULT1A1, GSTT1 and GSTM1 in Early Acute Leukemia**

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**Background:** Infant leukemia (IL) has a unique profile characterized by the high frequency of MLL rearrangements (MLL-r). A significant association between maternal use of estrogen during pregnancy and IL was recently observed and this finding deserves further investigations. Estrogen, pesticides, alcohol or tobacco substances are bio-transformed into either electrophile molecules that tend to be depurated or DNA-damaging compounds. These mechanisms may induce somatic MLL-r in childhood leukemia. In this context, sulfotransferase (SULT) sulfonates estrone to inactive estrone sulfate. The c.638G>A SULT1A1 (rs9828681) and c.667A>G SULT1A1 (rs1801030) decreases the enzyme activity and thermo stability. Hence, this study aims at investigating the SULT1A1 polymorphism frequency in early childhood leukemia (lymphoblastic, ALL and myeloid, AML). We also estimated the joint effect of GST genotypes on the risk of acute leukemia.

**Material and Methods:** Genotyping comprised 551 children (%)<24 month-old), 97.8% (256 samples of age-matched control group. Childhood leukemia samples were ascertainment from 2000 to 2010. The region of SULT1A1 gene (130bp) bearing both variant genotypes has been amplified; then PCR products were pyrosequenced to identify the specific genotypes (PyroMark Q2, QIAGEN). GSTT1 and GSTM1 null genotypes were also investigated in multiplex PCR reaction including a control gene with 312 bp (CYP1A1). Differences in the genotype distribution between patients and controls were assessed by logistic regression analysis by the software GraphPad Prism 5.

**Results:** The allelic frequency related to c.638G>A SULT1A1 variant was set at 30.6% and 31.7% in cases and controls, respectively. The c.667A>G SULT1A1 variant showed a lower frequency and the G allele was present in 10.3% of cases and 15.1% of controls. The absence of GSTT1 gene was found in 26.2% of cases and 30.1% in controls, while GSTM1 null genotype occurred in 40.6% and 39.8% of cases and controls, respectively. GSTT1 and GSTM1 null genotypes have not been associated with childhood leukemia risk. SULT1A1 c.667G (OR, 0.65, 95% CI, 0.43–0.98) was associated with protection. Age-depend association was observed in the c.667A>G SULT1A1 in children aged 13 <24 months-old (OR, 0.34, 95% CI, 0.17–0.70). When compared acute leukemia with or without MLL-r with controls, the SULT1A1 heterozygous children were prone to have acute leukemia without MLL-r (OR, 0.38, 95% CI, 0.17–0.85). Taken together, c.667A>G SULT1A1 variant, GSTT1 and GSTM1 null genotypes were associated as a protective factor in IL (OR, 0.21, 95% CI, 0.06–0.76).

**Conclusions:** Inconsistent found of the biological effect of c.667 A>G SULT1A1 variant, GSTT1 and GSTM1 null genotypes regarding the hypothesis that the risk of early childhood leukemia caused by maternal exposure to environmental toxicants should be further explored.

**Sunday 8 – Tuesday 10 July 2012**

**Poster Session: Prevention and Early Detection**

**[1176] Biologic Features and Prognosis of Ductal Carcinoma in Situ Are Not Adversely Impacted by Large Body Mass**

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**Background:** Obesity is associated with adverse biologic features and poor outcome in patients with invasive breast cancer. The relationship between obesity and prognosis in patients with DCIS has not previously been evaluated and was the subject of this study.

**Materials and Methods:** From 1996 to 2009, body mass index (BMI) was recorded at initial diagnosis for 1,885 patients with DCIS treated at our institution. Patients were categorized as obese (BMI ≥30 kg/m²), overweight (BMI 25 to <30 kg/m²), or of normal weight or underweight (BMI <25 kg/m²). Logistic regression was used to examine associations between BMI and patient, clinical, and pathologic features and treatment. Local regional recurrence was calculated using the Kaplan–Meier method. All statistical tests were 2-sided.

**Results:** Of the 1,885 patients, 514 (27.7%) were obese, 510 (27.5%) were overweight, and 831 (44.8%) were normal/underweight. In multivariate analysis, overweight and obese patients were significantly more likely to be African American (odds ratio (OR), 3.93) or Hispanic (OR, 1.44), be postmenopausal (OR, 1.63), have diabetes (OR, 4.60), have estrogen-receptor-positive DCIS (OR, 1.39), and present with a radiologic abnormality rather than clinical symptoms (OR, 1.35). At a median follow-up time of 4.96 years (range, 1.0–14.34 years), no significant differences in local recurrence rates were detected based on patients’ initial BMI category. Furthermore, there was no significant difference in risk of recurrence between diabetic patients receiving metformin or not.

**Conclusion:** Higher BMI is not associated with adverse biologic features or prognosis in patients with DCIS.

**[1177] A Randomized Controlled Trial of Diet, Physical Activity and Breast Cancer Recurrences – the DIANA-S Study**

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**Background:** Breast cancer (BC) incidence has increased steadily over the past decades, but BC mortality is declining, suggesting a benefit from early detection and more effective adjuvant treatment. Therefore, BC survivors are constantly increasing, and research investment for the identification of modifiable risk factors associated with recurrences have to be increased too. Obesity, the metabolic syndrome, and other conditions liked to insulin resistance, such as high serum levels of insulin and testosterone, affect BC progression and prognosis. It has been suggested that calorie restriction may reduce BC recurrences. The WINS randomized trial of low versus high total fat consumption in BC patients showed a significant 24% reduction of recurrences over 5 years associated with weight reduction. Previous DIANA trials showed that an insulin lowering diet, significantly decreases body weight, testosterone, insulin, and the bioavailability of growth factors in both healthy women and BC patients. A significant decrease in insulin resistance and testosterone also occurred in a randomized trial of moderate physical activity.

**Poster Session: Prevention and Early Detection**
We are now conducting a multicenter randomized controlled trial, the DIANA-5 study, to test the efficacy of dietary change and physical activity to prevent recurrent breast cancers at high risk because of high serum testosterone (>0.4 ng/ml), or insulin (>50 pmol/L), or the presence of metabolic syndrome. In these patients the estimated 5-year cumulative recurrence rate is 30%.

Patients and Methods: 2000 BC patients at high risk of recurrence were being randomized in two groups: 1000 receive the WCRF Decalogue for the dietary prevention of cancer; 1000 receive an active support (kitchen courses, physical activity classes, and common meals). Compliance is being monitored through weight and hormonal-metabolic change. The main analysis will be by intention to treat.

Results: Among 2076 BC patients currently recruited, we have randomized the 1417 at high risk (708 in the intervention and 709 in the control group.). At baseline the two groups were comparable for all the parameters under study. Preliminary results on 778 women examined after one year, showed a greater and significant reduction of body weight (−2.4 vs −1.0 kg, p = 0.03) and triglycerides (−19.7 vs −9.3 mg/dl, p = 0.02) in the intervention group compared with controls.

Conclusions: Preliminary results suggest that the lifestyle intervention is efficient. The study is currently in progress.

### 1178 Estimating Concentration of Chromophores in Tissue Phantom to Diagnosis of Cancer by Using Back Reflection Spectroscopy

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Introduction: Visible and infrared light spectroscopic methods have been investigated for diagnosis of cancerous tissues within the last two decades. Diffuse back reflection measurements of light in tissue can provide information about scattering and absorption properties of the tissues. Therefore, estimated concentration of the tissue chromophores based on the spectroscopic measurements has potential be used as a diagnostic parameter. Especially, obtaining concentration of endogenous chromophores such as hemoglobin, melanin, water and exogenous chromophores such as chemotherapy drugs may improve diagnosis of the cancerous tissues. Moreover, measurements of chromophores' concentration by the spectroscopic methods have advantages such as being noninvasive and real-time. In this study, a spectroscopic method has been developed to diagnose cancerous tissues by estimation concentration of the tissue chromophores and tested using tissue phantoms.

Material and Methods: In this study, a new spectroscopic model was developed to determine the concentration of chromophore molecules in tissue-like media by using the back reflection spectrum. Tissue phantoms have been prepared using Intralipid, water and indocyanine green (ICG) which is used as chromophore. Spectroscopic measurements on the tissue phantoms were carried out using a miniature spectrometer, a backscattering optical fiber probe consists of one illumination fiber surround by six detector fibers with a core diameter of 400 micrometer, a halogen tungsten light source and a laptop. All the reflectance spectra were acquired by inserting tip of the probe nearly 1 mm into the phantoms. Monte Carlo simulations of the experiments were performed. Simulations were used together with the experimental results to estimate concentration of the chromophore of the tissue phantoms.

Results and Discussion: Outcomes of the study were used to estimate absorption and reduced scattering coefficients of tissue phantoms using single back reflection spectrum. Scattering and absorption coefficients were estimated with an average error of 7.05% and 7.8% respectively. As a result, the concentration of ICG was calculated with 7.6% error.

Conclusion: The developed method has potential to be used in diagnosis of cancerous tissue in real-time and non-invasively based on the estimation of the tissue chromophores.

### 1179 Recreational Physical Activity and Mammographic Density in a Cohort of Postmenopausal Norwegian Women

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Background: Mammographic density (MD) is a strong risk factor for breast cancer and its treatment has the potential to noninvasively affect sexuality. The literature suggests that gynecological cancer survivors experience a number of sexual concerns that, in many cases, go unaddressed by health care providers (Abbott-Anderson & Kwekkeboom, 2012; Tierney, 2008; Hordern & Street, 2007). Common measures used in research about sexuality and gynecological cancer focus primarily on sexual dysfunction with items largely based on DSM criteria (e.g., Female Sexual Function Index, Changes in Sexual Function Questionnaire). Currently available measures do not address cancer survivors’ perspectives on sexual concerns beyond physical dysfunction. The Sexual Concerns Questionnaire-Gynecological Cancer (SCQ-GC) was developed to address this gap. The 48 SCQ-GC items were derived from a review of quantitative and qualitative literature that identified sexual concerns reported by gynecological cancer survivors. Items include concerns in physical, psychological, and social dimensions of sexuality, as well as items about communicating sexual concerns with the health care provider. The purpose of this study is to evaluate the content validity of the SCQ-GC.

Materials and Methods: The study uses a descriptive design. A purposive sample of 20 gynecological cancer survivors and a panel of 8 experts experienced in providing health care to gynecological cancer survivors are asked to evaluate the relevance and clarity (content validity) of each item in the SCQ-GC using a 4-point Likert-type scale and to provide suggestions for revision, including any missing sexual concerns that should be added.

Results and Discussion: To date, only panel of experts were asked to participate in the study. The panel of experts included experts in gynecological cancer as well as experts in oncology who are involved in offering comprehensive care to women with gynecological cancers.

Conclusion: Future research is planned to continue psychometric testing of the SCQ-GC in a larger sample of gynecological cancer survivors. It is anticipated that such research of sexual concerns developed from the survivor’s perspective may aid in the development of interventions to address a broader range of sexual concerns for gynecological cancer survivors.

### 1180 Dose-response Curves of Bleomycin Activity on in Vitro Genomic DNA for Dosimetric Purposes

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Background: Bleomycin is a glycopeptide that causes DNA strand breaks, used as anti-neoplastic agent in the treatment of diseases such as solid tumors and lymphomas. It has the capacity of produce free radicals on DNA in the same way as ionizing radiation. This study aims to investigate the in vitro clastogenic effect of this molecule using genomic DNA as a molecular model with the intention of establish dose-response curves for dosimetric purposes.

Materials and Methods: Genomic DNA: It was prepared from S. cerevisiae (WS8105–1C) cultures by phenol-chloroform extraction and ethanol precipita-
DNA Methylation in Lung Cancer Primary Tumor as a Biomarker in Eleven-gene Signature of Lung Biopsy Specimens for Cancer Diagnosis.

In this study, we evaluate the promoter methylation status of eleven tumor suppressor genes (CDH1, APC, FHIT, DAPK, SFRP1, GNAS1, P16, 6 (21%); GSTP1, 1 (3.5%); SOCs, 1 (3.5%); HFT 0 (%)).

High frequency of methylation at some of the above tumor suppressor genes suggests that the detection of these changes may help determine cancer susceptibility and early diagnosis. Additional studies are aimed to detect these changes in Circulating Tumor DNA and develop a panel of biomarkers for lung cancer early detection.

Results: Bleomycin induced DNA degradation that was increased with the increment of time of exposure and with the dose. The images of the gels analyzed showed that the DNA damage was about 20% of degradation at high bleomycin doses (0.45 UI/ml) and 24 hours of exposure. Longer exposure period (72 hours) induced a 30% of DNA degradation at a dose of 0.15 UI/ml, and a 50% of degradation at 0.45 UI/ml. Results showed a dose-response relationship that can be used to estimate the effective dose of bleomycin analyzing the DNA degradation obtained.

Conclusion: Belomycin is a genotoxic agent that can induce DNA degradation time and dose dependent. The optimization of dose-response curves, like in this study, permits the evaluation of the effective dose of exposure to a better evaluation of the antineoplastic activity based on the study of DNA degradation; which could be used for dosimetric purposes.

DNA Methylation in Lung Cancer Primary Tumor as a Biomarker for Cancer Diagnosis.

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Lung cancer is the leading cause of cancer deaths in the world among both men and women because most of them are normally diagnosed in late stage. Detection of lung cancer at earlier stages could potentially increase survival rates by 10 to 50-folds. Lung cancer screening often reveals more benign conditions that require invasive testing and expose people to unnecessary risks. Therefore safer diagnostic tests are required to detect cancer early. DNA methylation is a highly characterized epigenetic modification of the human genome that is implicated in cancer. Aberrant hypermethylation of the CpG island linked to some tumor suppressor genes is acquired during tumorigenesis. Some of these genes have been considered promising DNA methylation biomarkers for early cancer diagnostics. PCR-based methods that use sodium bisulfite-treated DNA as a template is generally accepted as the most analytically sensitive and specific techniques for analyzing DNA methylation at single loci. As the most analytically sensitive and specific techniques for analyzing DNA methylation biomarkers for early cancer diagnostics, PCR-based methods of the human genome that is implicated in cancer. Aberrant hypermethylation that the expression of 3 oncogenes bcl-2, k-ras and h-ras were significantly high (p < 0.05) in surgically removed tumors as well as biopsy specimens compared to adjacent normal tissues. The differential expressions of these 3 genes were strikingly higher in cigarette users than non-smokers.

Conclusion: The gene expression profiling techniques using real-time qRT-PCR is, efficient, consistent and reliable to examine small biopsy specimens. Considering the overall results of this study, it can be concluded that, lung tissue abnormalities other than cancer could also cause the elevation of some oncogene expression. This study further verifies that smoking can definitely cause oncogenic mutation, activation and/or amplification; which is now a globally proven fact. Overall, our data show the feasibility of a relatively simple diagnostic test for lung biopsy specimens.

Dietary Patterns and Postmenopausal Breast Cancer Survival – a Prospective Patient Cohort Study

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Introduction: Research on the association between dietary patterns and breast cancer survival is very limited.

Materials and Methods: We assessed the association of pre-diagnostic dietary patterns with survival and recurrence in a prospective cohort study in Germany. Postmenopausal breast cancer patients were diagnosed between 2001 and 2005, and vital status, causes of death, and recurrences were verified through the end of 2009. Food frequency questionnaire data referring to the year before diagnosis were available for 2,522 patients, and principal component factor analysis was used to identify dietary patterns. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated with Cox proportional hazards models, stratified by age at diagnosis and study center and adjusted for relevant prognostic factors.

Results: Two major dietary patterns were identified: ‘healthy’ [high intakes of vegetables, garlic/onions, oil and vinegar dressing, mayonnaise (primarily from salad dressings), and red meat] and ‘unhealthy’ [high intakes of red meat, processed meat, and deep-frying fat and low intake of fruits]. Increasing consumption of an ‘unhealthy’ dietary pattern was associated with a significantly increased risk of overall mortality (highest versus lowest quartile: HR = 1.48, 95% CI: 1.05, 2.07) and non-breast cancer mortality (HR = 3.80, 95% CI: 1.83, 7.86). No associations with breast cancer-specific mortality (HR = 1.09, 95% CI: 0.73, 1.61) and breast cancer recurrence (HR = 1.18, 95% CI: 0.81, 1.71) were found. The ‘healthy’ dietary pattern was not associated with any of the outcomes.

Conclusion: In conclusion, increasing intake of an ‘unhealthy’ dietary pattern may increase the risk of overall and non-breast cancer mortality.
**Nordic Lifestyle Intervention Trial on Prostate Cancer**

**Introduction:** Prostate cancer (PC) is highly prevalent in elderly European men. The incidence is increasing. Insulin resistance and hyper-insulinemia are associated with high risk of aggressive PC and increased mortality. We hypothesize that vigorous exercise and high whole grain rye intake reduce detrimental metabolic effects on PC progression by effects on insulin secretion and insulin sensitivity. We will explore processes involved in PC progression in a Nordic randomized, clinical trial in Sweden, Iceland and Denmark. By employing a comprehensive assessment of the metabolic profile and PC progression, in addition, we will identify lifestyle factors with co-beneficiary effects such as higher physical well-being and life quality and lower risk of obesity-related diseases, and thereby contribute to a healthy ageing. The comprehensive and detailed information on diet and lifestyle through questionnaires and assessments, laboratory analyses on urine and blood samples and prostate tissue, and the access to cancer registry data on health outcome in the Nordic countries enable us to conduct health research beyond the state of the art, which may be used to define effective public health recommendations and disease prevention guidelines pertinent to the European male population. Firstly, we are conducting a feasibility study in Denmark.

**Material and Method:** For the on-going feasibility study, 24 Danish men are enrolled and randomly assigned to an intervention group (16 men) or a control group (8 men). The participants are 45–70 years of age and newly diagnosed with early-stage PC. The intervention group is prescribed 180g of whole grain rye per day and vigorous exercise 3 times 45 minutes per week. No prescriptions or restrictions are made for the control group. Duration of intervention is 6 months with follow-up 12 months after baseline. Blood and urine samples are collected at baseline, month 3, 6 and 12 and prostate biopsies at baseline and month 6. Effects on metabolic profile and cancer progression are assessed by measurements on anthropometrics, oral glucose tolerance tests, metabolomics, prostate specific antigen and glycated haemoglobin in blood and urine. Physical well-being and life quality are evaluated by questionnaires and qualitative interviews.

**Results and Discussion:** Collection of data and biological samples are ongoing until October 2012. Laboratory and statistical analyses are conducted during 2013.

**Conclusion:** No conclusions are yet to be made.

**Chemoprevention in Head and Neck Cancer (HNC)**

**Background:** Natural dietary agents have drawn great attention for chemoprevention because of their low/non-toxic nature and multi-targeted effects. However, low bioavailability and/or efficacy of most natural compounds as single agents pose challenges for potential clinical application. Thus, combinations of natural compounds with synergistic effects are warranted. We have screened many different combinations of natural compounds and found that combination of green tea EGCG and resveratrol has strong synergistic effects in cell growth inhibition.

**Methods:** Malignant and premalignant HNC cell lines were used in the study. Propagation of V600E mutant cells were conducted to tolerate cell cycle and apoptosis, respectively. CalcuSyn software was used to measure combination index. Expression of individual proteins involved in relevant molecular pathways was examined by Western blotting. Retrovirus-mediated gene transfer was performed to overexpress constitutively active (CA-)AKT and nude mouse xenograft model for in vivo study.

**Results:** Combination of EGCG (30–80 μM) and resveratrol (15, 20 μM), at which single agents induced minimal apoptosis (<15%), strongly increased apoptosis (60–80%). Combination index values were between 0.4–0.7, indicating high synergy. Interestingly, a premalignant oral leukoplakia cell line was sensitive to relatively lower doses of the combination (5 and 10μM resveratrol and 30 μM EGCG) than the transformed HuMed cell line. The combination of EGCG and resveratrol strongly inhibited p-ERK and pERK along with several downstream targets including p-MTOR, p-S6 and p-4EBP1. Overexpression of CA-AKT significantly inhibited apoptosis induced by the combination (p = 0.003). The combination of EGCG and resveratrol also strongly inhibited p-AMPK, a kinase that regulates autophagy (a cell protective mechanism) during energy stress. Furthermore, strong inhibition of anti-apoptotic Mcl-1 and survivin by the combination was also observed. Finally, the combination of EGCG and resveratrol significantly inhibited the growth (Ki67/proliferation (K67) of HNC tumor xenografts bearing the HNC Tu212 cell line, as compared with either single agent.

**Conclusions:** Our study has identified a novel combination of two natural dietary agents, EGCG and resveratrol, which induces synergistic apoptosis in both premalignant and fully transformed HNC cells. Combination of these agents inhibit signal transduction pathways, among which AKT-mediated pathways are major effectors. Since AKT is over-activated in premalignant lesions of head and neck and is strongly inhibited by the combination of EGCG and resveratrol, our study provides an important rationale for the future clinical development of this combination for chemoprevention of HNC.

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**WWTR1 and CYR61 Are Early Prognostic Markers of Barrett’s Esophagus Malignant Progression**

**Background:** The metaplasia of the esophagus known as Barrett’s esophagus (BE) is seen as a major risk factor for the development of esophageal adenocarcinoma (EA), alongside obesity and gastroesophageal reflux disease (GERD). But accumulating data is now questioning if all BE metaplastic transformations are truly associated with a malignant transformation or if BE can actually protect against GERD. In addition, recent epidemiological data has pointed to a very low risk of BE progression to EA. It is thus imperative to develop better methods to stratify patients with a real risk of BE neoplastic progression. In the present work we set to define early molecular biomarkers of BE malignant progression, through the usage of an innovative bioinformatics framework applied to publicly available global transcriptome data associated with the progression of BE to EA.

**Material and Method:** Three publicly available datasets on Affymetrix® H133a microarray platform were retrieved and analyzed with a set of functions implemented in the R Statistical Computing software complemented with Biocductor. Briefly, raw data .cel files were summarized with the affy package, followed by normalization with frma package. Biomarker prioritization included: (1) usage of Expression Barcode 2.0 (http://rafalab.hubud.harvard.edu) and (2) filtering using differential expression analysis with limma package. Selected candidate genes were validated by qRT-PCR on RNA extracted from formalin-fixed paraffin embedded (FFPE) archived samples.

**Results and Discussion:** The bioinformatics framework outlined above applied to the mined BE transcriptome allowed us to identify a set of 12 up-regulated genes that under the most conservative criteria can distinguish a BE that is likely to progress from a BE that will not progress to EA. A second filter, followed by qRT-PCR validation of selected biomarkers further trimmed the list of candidates to 2 markers (WWTR1 and CYR61). qRT-PCR on time-series FFPE RNA of EA-progressed and EA-free BE samples showed that these genes are already up-regulated many years before the development of EA as compared to patients who never developed EA. Furthermore the up-regulation of such markers is already detectable in normal esophageal mucosa of early biopsies of progressed patients.

**Conclusions:** Our results suggest that alterations with prognostic value can be detected very early in BE and in histological normal mucosa and thus impact on EA diagnosis and treatment.

**Detection of Neuronal Cancer Cells by Raman Spectroscopy**

**Background:** Raman spectroscopy (RS) is an optical spectroscopy method which is label-free and can be used even in cell culture media. In RS laser light is used to excite molecular vibrations which lead to a shift in frequency of the emitted light and can be detected by a spectrophotogram. The resulting spectra contain detailed chemical information about the sample. Like a ‘fingerprint’ this information can be used to identify cell populations and cancer cells or the cellular response to drug treatment. For cancer research and clinical practice a fast and reliable method is needed to identify cancer cells in cell suspensions and tissue sections. In this work we describe Raman analysis of 3D cultured neuronal tissue in combination with Glycobioloma and neuronal cells.

**Material and Methods:** Tissue engineered neuronal models consist of neuronal cells and neuronal stem cells seeded on a semipermeable membrane.
Diet and DNA Damage

Investigation of Raman Spectroscopy and Optical Coherence Tomography to Aid in Diagnosis of Oral Cancer

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Introduction: The diagnosis of oral cancer (OC) currently requires surgical biopsy and histological analysis, which is invasive and time consuming. The fact that in most cases the detection of the disease can only be possible in an advanced stage leads us to look for biomarkers that could detect it early, and contribute to a greater knowledge about the disease process. Thus, the aim of this work was to evaluate the silica genotoxicity through comet assay, and catalase activity, as possible markers of early-stage disease detection.

Aim: To investigate two novel methods for the diagnosis of OC. The first of these is Raman spectroscopy, a technique able to determine the specific chemical bonds present in a sample and their relative quantities. The second technique is optical coherence tomography (OCT), a non-invasive imaging technique which is commonly used in ophthalmology. Here we investigate the potential of this technique to visualise the oral epithelium.

Materials and Methods: A DXR Raman microscope was used to obtain spectra from healthy, pre-cancerous and cancerous tissue sections. These sections were from either tissue engineered models of OC and the oral mucosa or from archived patient samples. Raman spectra (RS) were also obtained from different cell lines (cancer and dysplastic), healthy oral cells isolated from excised patient tissue and tissue engineered models of mucosa and OC. Tissue engineered models were cultured as described previously (Colley et al., Br J Cancer 2011). OCT images of tissue engineered models and healthy human volunteers were obtained using an OCT device.

Results and Discussion: It is possible to obtain RS from tissue sections, cells and tissue engineered models, and differences were observed between normal and cancerous samples. OCT could non-invasively image over 500 μm into oral tissue in real-time. It was possible to identify the epithelial-connective tissue architecture and superficial connective tissue structures such as blood vessels and minor salivary glands.

Conclusion: RS of in vitro samples and ex vivo sections show variations between healthy tissue and cancerous tissues which could have potential as a diagnostic aid for OC. Further studies are required to determine the potential use in patients. OCT images show differences between tissue engineered models of OC and normal mucosa. Real-time images of healthy volunteers were easily obtained and showed the epithelial connective tissue boundary. Studies in patients with potentially malignant lesions will further test the clinical benefits of OCT.

1193 Searching for Biomarkers in Silica Early Exposure

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Introduction: Silica is formed by the elements silicon and oxygen under high pressure and heat. It can be found in the amorphous and crystalline forms. Inhalation of free crystalline silica results in a spectrum of lung diseases known as silicosis. Silicosis is characterized by lung fibrosis and it appears to be a major cause of occupational lung disease in exposed workers. There is a latency of 10 to 30 years, although the disease can develop earlier in workers exposed to high quantities of fine silica dust over a relatively short period of time. Identification of silicosis at a population level in workers exposed to silica containing dust has until recently been carried out by conventional chest radiographs, classified according to the International Labour Organization guidelines. Moreover, the interpretation of standard radiographs in accordance with international agencies, has proven problematic in regards to the reliability of an inter and intra reading. Silicosis is a disease that has no cure, and can result besides its severe symptoms, tuberculosis and lung cancer associated. Moreover, the whole process of the disease is still unknown. The fact that in most cases the detection of the disease can only be possible in an advanced stage leads us to look for biomarkers that could detect it early, and contribute to a greater knowledge about the disease process. Thus, the aim of this work was to evaluate the silica genotoxicity through comet assay, and catalase activity, as possible markers of early-stage disease detection.

Materials and Methods: Mice were treated with silica instillation (10 mg/50 μl NaCl solution) and 3 and 7 days after, blood samples were analyzed by Comet assay, besides catalase activity determination.

Results and Discussion: Comet assay showed enhanced level of DNA damage (p = 0.0102), in treated cells as compared with control, after 7 days from the exposure, indicating that the DNA damage could be due to stress caused by silica instillation. There was also significant difference in the level of DNA damage between the exposed groups of 3 and 7 days showing a possible increased effect. Moreover the statistical analysis showed no difference between control and exposed groups after 3 days (p = 0.0817). Since the p value is found near the significance level, a new assay, with increasing in sample size, could clarify this issue. The catalase activity showed no significant difference between control and treated animals (p = 0.05). This result could be due to the fact that the concentration of peroxide, generated in the inflammatory process of silica exposure, did not reach the Km of the enzyme, which would be necessary to observe differences in its activity.

Conclusion: Treatment of mice with silica instillation was observed mainly after 7 days from exposure, when compared treated and control groups. So, comet assay appears to be a promising assay to be used in early-stage silicosis detection.

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Introduction: Malignancies of gastrointestinal tract (GIT) are the most rapidly growing problem. Development of cancer results from a combination of environmental factors and an accumulation of specific genetic alterations. Researchers indicate that the risk of cancers can be reduced through primary prevention. Numerous, known risk factors can be potentially avoided, therefore behavior predisposing to GIT malignances became the most promising target of interest for practitioners and researchers. The aim of the project was to explore the state knowledge about risk factors, primary prevention and early detection of GIT malignances in the urban and rural population of the Lublin province (eastern Poland).

Materials and Methods: The study involved 1352 volunteers aged 24 to 87 years. The research was representative for the rural and urban population. The research tool was an originally designed questionnaire consisting of 12 questions. Our responders were patients presenting with different reasons to their family doctor. Interview and fill out the survey was conducted by a person in each of the primary care practices participating in the study. The study was reviewed and approved by the institutional ethical board of Medical University of Lublin KE-0254/73/2011. Survey results were statistically analyzed by Statistica (Statsoft, version 9.0, USA). Dependences between the variables were analyzed by χ² Pearson’s test and correspondence analysis.

Results and Discussion: Our study showed that the population of the Lublin province has a low awareness and knowledge about prevention of GIT malignances. We found the relationship between the awareness of GIT malignances and the place of residence at the significance level p < 0.001. Thus, the place of living has a strong influence on awareness of GIT malignances than the education level.

In the rural areas most people use the TV and the general practitioner as a source of information about cancers. In the urban areas the most popular source of cancers information is the leaflet.

Conclusion: Different health promotion programs should be designed for urban and rural populations as well as various routes of entry of the programs.
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