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Parallel Sessions (Proffered Papers)

Parallel session 1: Biomarker Discovery

PP01
MARK4: A TRICKY BALANCE OF L AND S ISOFORMS RULES GLIAL DIFFERENTIATION AND GLIOMA PROGRESSION

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The Microtubule Affinity-Regulating Kinase 4 belongs to a kinases family that phosphorylates the Microtubule[MT]-Associated Proteins, causing their detachment and increasing MT dynamics. MARK4 encodes two alternative spliced isoforms, L and S, which expression is differentially regulated in human tissues. In normal brain the predominant expression of MARK4S has been related to a putative role in neuronal differentiation; the L isoform has been conversely found highly expressed in neural progenitors and in gliomas, as well as in hepatocarcinoma cell lines, highlighting a general role in neoplastic transformation.
The current study aimed at better defining the role of MARK4 L and S in gliomagenesis. The expression levels of these isoforms were investigated by Q-PCR and WB on 50 gliomas (low and high grade tumors and cell lines) and 8 cancer stem cell lines (CSC), in addition to normal brain, neural stem cells (NSC) and neural progenitors.
Array-CGH and mutation analysis failed to reveal any genomic alteration. Expression profiling of MARK4 L and S in glioma highlighted a predominant expression of the L isoform in parallel with a significant decrease of the S levels, that correlate with tumor grading, suggesting a change in the ratio between the two isoforms during glioma progression. These findings let us hypothesize an involvement of alternative splicing in regulating the relative expression of the two isoforms in normal cells, that could be altered in gliomagenesis.
Evaluation of MARK4 expression levels in CSC vs NSC showed that the S isoform is slightly detectable; conversely, a significantly higher expression of MARK4L is estimated in a subset of CSC, strengthening a role of MARK4L in glioma tumorigenesis.
We found that the anti-MARK4L antibody stains *in vivo* cells in human and mouse embryonic ventricular zone and in mouse adult sub-ventricular zone (SVZ), well known regions of stem cells localization, thus delineating MARK4L as a stemness marker with potential tumorigenic ability. This evidence, together with the retrieval of MARK4L altered expression in glioma, reinforces the stem cell hypothesis on the origin of glial tumor, at least glioblastoma (GBM). We are currently investigating paraffin-embedded glioma sections to correlate MARK4L localization to the region of tumor formation, also by evaluating MRI features of GBMs in relation to the SVZ.
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PP02
IDENTIFICATION OF *PTPRJ* AS A NOVEL GENE INVOLVED IN COLORECTAL CANCER PREDISPOSITION

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In the majority of familial colorectal cancers (CRC) the disease causing genetic factors are still unknown. In order to identify novel CRC predisposing genes we carefully selected 32 independent index patients with microsatellite-stable CRC. All patients were highly suspected for hereditary CRC because of their young age at diagnosis and/or clear positive family history for CRC. We employed genome-wide copy number profiling using high-resolution SNP-based array CGH on normal tissue DNA and identified a partial duplication encompassing the 5' region of the protein tyrosine phosphatase gene *PTPRJ* in an early-onset CRC patient. *PTPRJ* is a tumor suppressor gene that was previously reported for CRC susceptibility in mouse, but mutations or copy number changes in human have never been reported in relation to CRC predisposition, nor as regions of polymorphic copy number variation. A targeted copy number screen of a large cohort of familial CRC patients (n=1500) and controls (n=1050) revealed a second but clearly different partial duplication affecting the *PTPRJ* gene in an early onset CRC patient. We found that in at least one of the two patients the duplication was in tandem (head to tail orientation), resulting in the expression of a fusion transcript in which exons 2-11 were duplicated. Furthermore, we were able to show that expression of *PTPRJ* in the patient was mono-allelic, originating only from the wild type allele, indicative for a loss-of-function scenario. From our results we conclude that *PTPRJ* serves as a novel candidate CRC susceptibility gene.

PP03
IDENTIFICATION OF PRION PROTEIN AS A CELL SURFACE CANDIDATE BIOMARKER FOR COLORECTAL ADENOMA-TO-CARCINOMA PROGRESSION

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Question: Early diagnosis of colorectal cancer (CRC) is a realistic approach to reduce its high mortality rates. Currently available methods for early detection of CRC do not distinguish the 5% of adenomas expected to progress into CRC (high-risk adenomas) from adenomas with low risk of progression. We aimed to identify cell surface protein biomarkers that can be targeted for molecular imaging and discriminate low-risk adenomas from high-risk adenomas and CRC.
Methods: Genome-wide mRNA profiling revealed genes with increased expression in CRCs compared to adenomas (Carvalho et al., 2009). To identify the subset of genes encoding plasma membrane-bound proteins, extracellular domains of cell surface proteins of five CRC cell lines were biotinylated, isolated, and analyzed by in-depth proteomics using gel electrophoresis and nanoliquid-chromatography coupled to tandem mass spectrometry. Cell surface expression was confirmed by FACS analysis and immunohistochemistry.
Results: In total 2609 proteins were identified in the cell surface fractions. Of these, 31 candidate biomarkers were selected based on protein identification in at least four cell lines, a predicted (trans)membrane location, and increased mRNA expression in CRC compared to adenomas ($p<0.05$). For one candidate, the cellular prion protein PrPc, increased expression in a series of CRCs compared to adenomas was verified by immunohistochemical evaluation.
Conclusions: Our strategy successfully yielded cell surface candidate biomarkers for molecular imaging of adenoma-to-carcinoma progression, exemplified by identification of PrPc. Moreover, this study illustrates that panels of biomarkers will be required to cover detection of the full spectrum of the molecularly heterogeneous group of high-risk adenomas and CRCs.

PP04
FAST AUTO-QUANTIFICATION OF IHC BIOMARKERS (BAK AND BAX) AND DISCOVERY OF NOVEL PROGNOSTICALLY SIGNIFICANT TUMOUR SUBSETS IN NSCLC

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Tissue microarray is a high throughput tool to identify new diagnostic and prognostic markers and targets in human cancers, based on the assessment of immunohistochemistry (IHC) or fluorescent in-situ hybridization (FISH). TMAs complements discovery

studies by confirming results on large numbers of primary tumour cases. Each tissue core is assessed by two independent pathologists. Manual scoring is time consuming, error-prone and subjective. Moreover, human eye is not sensitive to fine differences in colour intensity and semiquantitative mode of manual scoring produces limited information for analysis, which seriously restricts utility of FISH and IHC.

Lung cancer is the leading cause of cancer-related death worldwide, and non-small cell lung cancer (NSCLC) represents around 80-90%. We have developed a fully automated and computational efficient method that allows rapid, continuous and quantitative analysis of TMAs based on IHC, and investigated two novel biomarkers (BAK and BAX) in NSCLC patient survival. The automated method separates tumour cells from stroma and produces robust scores across TMAs as normalized density of the positively stained tissue of the tumour cells.

We stained four TMAs with 458 NSCLC positive tumour cores. All cores were independently scored by experienced pathologists. Using spearman rank correlation test, the automated scores were statistically significant correlated with the pathologist-based scores (all p-values < 0.01). Importantly, the automated analysis identifies four novel prognostically significant tumour subsets, which were not detected by traditional pathologist-based scoring. With Kaplan Meier analysis, the automated analysis shows that adenocarcinoma patients with low BAX have statistically significant worse survival ($p<0.001$) than other adenocarcinoma patients; adenocarcinoma patients with BAX and BAK both low have statistically significant worse survival ($p=0.008$). With Cox Regression analysis, automated analysis shows a relative risk of 2.811 ($p=0.001$, 95% CI 1.537-5.141) for adenocarcinoma patients with low BAX, and a relative risk of 3.749 ($p=0.015$, 95% CI 1.287-10.914) for adenocarcinoma patients with BAX and BAK both low. In summary, this approach empowers IHC for use in discovery of new diagnostic and prognostic targets in human cancers.

PP05
METALLOPROTEASE ADAM10 IS REQUIRED FOR NOTCH1 SITE 2 CLEAVAGE

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Notch receptors are type I transmembrane receptors composed of a large Notch extracellular domain and a transmembrane/intracellular domain held together by a heterodimerization domain. Notch signaling is activated by proteolytic cleavage in response to receptor-ligand interactions between adjacent cells. Ligand binding unfolds the Notch heterodimerization domain and facilitates two cleavages first at Site 2 (S2) removing the extracellular domain and next a transmembrane cleavage by γ-secretase at Site 3 (S3), releasing the Notch Intracellular Domain.

S2 cleavage of Notch1 is catalyzed by a metalloprotease and a rate-limiting step for S3 cleavage. To date the identity of the Site 2 cleaving metalloprotease in Notch1 signaling has been elusive. In contrast to studies in flies, where S2 cleavage is shown to be mediated by ADAM10, studies in mammalian cells indicate a role for ADAM17 in this process. Yet, the phenotype of mice knock-out (KO) for ADAM17 does not resemble a Notch1 KO phenotype, while the ADAM10 KO phenotype does.

Here we show that the metalloprotease ADAM10, but not ADAM17, is required for extracellular cleavage of Notch1 at Site 2 when stimulated with ligand. Interestingly, ligand independent Notch1 signaling does not fully rely on cleavage by the ADAM10 protease. Both genetic and pharmacological inhibition of metalloproteases still allowed Notch1 cleavage, indicating the presence of an unknown protease cleaving at Site 2. Since cancer-causing Notch1 mutations likely also depend on Site 2 cleavage for their activity, the identity of these alternative proteases has important implications for understanding Notch activation in normal versus cancer cells.

PP06
PROGNOSTIC SIGNIFICANCE OF THE HYPOXIA GENE SIGNATURE IN STRATIFYING NEUROBLASTOMA PATIENTS

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Neuroblastoma is the most common extracranial solid tumor in childhood and shows notable heterogeneity with regard to both histology and clinical behavior. Hypoxia, a local decrease of oxygen tension, is a critical determinant of tumor progression inducing angiogenesis, matrix degradation and inhibiting

apoptosis and cell differentiation. Therefore, hypoxia is related to poor prognosis in human cancer and it has a profound impact on neuroblastoma aggressive behavior. Our aim is to define the hypoxia signature from in vitro controlled system and to test its prognostic value on the gene expression profiles of a cohort of neuroblastoma patients.

*l*1-*l*2 regularization framework has been applied on gene expression profiles of 11 neuroblastoma cell lines to define the neuroblastoma hypoxia signature (NB-hypo). We applied k-means clustering on the expression level of the signature 62 probesets to segregate 88 neuroblastoma patients and subgroups obtained by common risk factors stratification. We analyzed the classes by Kaplan-Meier curves and log-rank test for overall survival (OS) and event-free survival (EFS). Multivariate Cox analysis was performed to define the predictive power of the signature.

The NB-hypo signature distinguished two groups of neuroblastoma patients classifying them as poor prognosis (21 patients), those having OS rate of 25.5% and EFS rate of 27.7%, and as good prognosis (67 patients), those having OS rate of 73.2% and EFS rate of 67.7%. The poor prognosis patients show an over-expression of the hypoxia probesets. Multivariate Cox analysis revealed that the NB-hypo signature is a significant independent predictor after controlling for commonly used risk factors. When applied to MYCN not amplified patients, the NB-hypo signature was capable to stratify patients with OS rate of 24.2% and EFS rate of 27.3% for the patients with poor prognosis, compared with OS rate of 81.4% and EFS rate of 74.8% for the patients with good prognosis.

We demonstrated that the NB-hypo signature is a significant prognostic factor capable of stratify neuroblastoma patients. Furthermore, we obtained the proof of principle that the approach of hypoxia genes selection from in vitro controlled tumor cell lines is a feasible method to identify specific contribution of the microenvironment to the tumor biology.

Parallel session 2: Cytometry and Virtual Pathology

PP07
CYTOMIC ANALYSIS OF CANCER STEM CELLS IN HUMAN MELANOMA CELL LINES

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Cancer stem cells (CSC), also known as tumor initiating cells, have been identified in a variety of human and mouse tumors through the use of known and novel stem-cell markers and by the performance of strict self-renewal and tumorigenicity assays. The expression of stem-cell markers and the isolation of stem-like cells have been recently described in melanoma.

In the present study, we have characterized CSC of 13 melanoma cell lines, five established from primary tumors and eight established from metastasis from different tissues. For this, we have analyzed through flow cytometry the expression of the cell surface markers CD20, CD34, CD90, CD117 (ckit), CD133 and the ABC transporters ABCB1, ABCB5 and ABCG2. Furthermore, we analyzed through flow cytometry the existence of the *side population* (SP), based on the efflux ability of this population (SP) to exclude the fluorescent dye Hoechst 33342.

Our results show disparities in the pattern of stem-cell markers. No cell line was $CD20^+$, two were $CD90^+$, three were $CD133^+$, five were $CD34^+$ and seven were $CD117^+$. All cell lines expressed at least one ABC transporter, being ABCB1 the transporter expressed in a higher percentage of the population (5.85% - 82.42%) while ABCG2 and ABCB5 were expressed in a smaller percentage of the population (0.50% - 7.90%). The presence of SP was found in seven cell lines (four of primary origin and three of metastasis). In addition, all the SP^+ cell lines expressed at least one ABC transporter. These results suggest that there is no relation between the cell line origin (primary tumor or metastasis) neither with the pattern of stem-cell markers expression nor with the existence of SP.

A study of the co-expression of these stem-cell markers within the same cells is necessary for a thoroughly comprehension of the melanoma stem cells.

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PP08
THE PROPORTION OF TUMOUR CELLS IS AN INDEPENDENT PREDICTOR FOR SURVIVAL IN COLORECTAL CANCER PATIENTS

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Background: The proportion of epithelial and stromal cells in tumours is thought to play an important role in the progression of epithelial malignancy. We aimed to determine whether the relative proportion of tumour (PoT) was related to survival in colorectal cancer.
Methods: The PoT at the luminal surface was measured by point counting using virtual tissue sections in a series of 145 colorectal cancer cases. The relationship of PoT to clinicopathological parameters including cancer specific survival was analysed. Modified receiver operating characteristic curves were used to determine the optimum cut off points to dichotomise the data for survival analyses.
Results: Tumours with PoT-low (≤47%) were associated with significantly lower cancer specific survival when compared to PoT-high (HR 2.087, 95% CI 1.088 to 4.003, p=0.024). Multivariate Cox regression analysis demonstrated that PoT was an independent prognostic marker when adjusted for age, T stage, N stage and extramural vascular invasion (p=0.017).
Conclusions: This study suggests that a low proportion of tumour cells within colorectal cancer is related to poor cancer specific survival. A relatively quick, inexpensive and well-established method such as point counting on diagnostic tissue sections could be used to identify a subset of patients who may benefit from adjuvant therapy.

PP09
LARGE-SCALE GENOMIC INSTABILITY IN COLON ADENOCARCINOMAS AND CORRELATION WITH PATIENT OUTCOME

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The purpose of this study was to evaluate the association between DNA content in colon adenocarcinomas and patient outcome. Tumours from 219 patients operated for colon adenocarcinoma were analysed using high-resolution image cytometry. Proteins involved in cell cycle propulsion (cyclins A, D1, D3 and E) and cell proliferation (c-Myc and non-membranous β-catenin) have previously been reported in the same cohort and were included in this study. The results were related to disease-free survival and to cancer-specific death. Patients with aneuploid tumours showed shorter relapse-free survival than patients with euploid tumours (univariate log-rank test, p = 0.004 and multivariate Cox regression model p = 0.009, HR 0.51, 95% CI 0.31-0.84). Also the risk of death from cancer was greater in patients with aneuploid tumours (log-rank test, p = 0.006 multivariate Cox regression model p = 0.014, HR 0.47, 95% CI 0.26-0.86). When analysing patients with Dukes stages A and B, nuclear expression of β-catenin was highly significantly associated with both shorter relapse-free survival (p < 0.005, HR 5.0, 95% CI 1.6-15.5) and cancer-specific death (p = 0.036, HR 6.9, 95% CI 1.1-42.1). DNA content in colon adenocarcinomas measured by image cytometry is an independent predictor of prognosis in our patients operated for colon adenocarcinoma.
In addition to this study we are in the process of analysing approximately 900 additional colorectal cancer patients, and these results will be presented as well.

PP10
DNA PLOIDY HETEROGENEITY IN ENDOMETRIAL CARCINOMA: COMPARISON BETWEEN CURETTAGE AND HYSTERECTOMY SPECIMENS

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Background: DNA ploidy has been reported to be a prognostic marker for patients with endometrial carcinoma. There has been little focus on whether performing this analysis on curettage vs. hysterectomy specimens affects the results. The prognostic role of DNA ploidy analysis performed in curettage specimens has been disputed.
Methods: In the present study, DNA ploidy and histological heterogeneity were evaluated by comparing curettage and hysterectomy specimens in 99 consecutive patients diagnosed with endometrial carcinoma. High resolution DNA ploidy image analysis and review of histological specimens were performed.
Results: The histological subtypes were identical in 77 (78%) and differed in 22 (22%) cases. The DNA ploidy results were concordant in curettage and hysterectomy specimens in 72 (72.7%) and discordant in 27 (27.3%) cases. Histological heterogeneity was associated with DNA ploidy heterogeneity (p=0.03). Based on histological heterogeneity, DNA ploidy-discordant cases were divided into two groups. One group (16.2% of cases) consisted of specimens with similar histology in curettage and hysterectomy, all of the endometrioid subtype. This group showed DNA ploidy discordance due to a DNA diploid peak in one specimen and an aneuploid peak (DI=1.05-1.2) in the other. The other group (11.1% of cases) consisted of cases with different histological subtype or grade and demonstrated a more pronounced DNA ploidy difference (diploid vs. aneuploid with DI>1.2).
Conclusion: Our results suggest that the DNA ploidy results of hysterectomy and curettage specimen are not identical. The difference observed, which we believe to reflect intratumoral heterogeneity, should be taken into account when applying DNA ploidy to endometrial carcinoma specimens.

PP11
AUTOMATIC SEGMENTATION OF CELL NUCLEI IN HISTOLOGICAL SECTIONS

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We have developed a method for automatic segmentation of cell nuclei from Feulgen stained tissue sections of prostate cancer. We have combined a gradient-validated local adaptive thresholding with an active contour model that features an optimized initialization and works within a restricted region to improve convergence of the segmentation of each nucleus.
A small number of samples seem to be a common characteristic of many papers on evaluation of accuracy in medical image segmentation. Our method was tested on a total of 30 randomly selected frames from 3 cases, comparing the results from the automatic algorithm to expert manual delineation of 924 cell nuclei.
Considering only cell nuclei larger than 2400 pixels (diameter ≈ 55 pixels, or 5.6 µm), the automatic method segmented about 10% more nuclei than the manual method, and about 75% of the manually segmented nuclei were also segmented by the automatic method. For each nucleus segmented both manually and automatically, the accuracy (i.e., agreement with manual delineation) was estimated. The mean segmentation sensitivity/specificity was 95/96%.
Correlation analysis indicated a very close relation between the area of the manual segment and the area of the automatic segmentation on the same cell nuclei. There was, however, a systematic bias towards a slightly larger manual nuclear area, consistent with a 1-2 pixel wide manual perimeter outside the automatically segmented nuclei.
Both bias and uncertainty in nuclear area will influence both average gray level and standard deviation of nuclear gray level, as well as estimated nuclear textural parameters. We have therefore quantified these parameters and discussed their relation to segmentation accuracy.
For cell nuclei that were only segmented by one of the methods, we checked for systematic differences in typical nuclear parameters. This was done by a three-way quantification of differences and biases in nuclear area, mean nuclear graylevel, and graylevel entropy.

PP12
HIGH PERFORMANCE COMPUTING FOR HIGH THROUGHPUT TISSUE MICROARRAY ANALYSIS

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Tissue Microarrays (TMAs) are used extensively in tissue-based research and biomarker discovery. Since multiple tissue samples can be analysed in a single assay, it potentially represents a rapid method for assessing and identifying new IHC markers. The major bottleneck however is that it still largely relies on visual scoring of tissue biomarkers which is time consuming and prone to error. Even when image analysis is applied to digitised TMA whole slide scans (virtual slides), the size of the images, the large number of tissue cores demands considerable processing power making the process slow on a standard computer.
This study aims to develop a high performance computing (HPC) approach for the automated image analysis of TMA virtual slides; as a means of removing subjectivity, significantly speeding up biomarker evaluation on TMAs, and developing a truly high throughput approach to TMA analysis.
Using a HPC cluster (Queen's University HPC Centre) containing >9000 processor cores, we developed an HPC platform dedicated for the rapid analysis of TMA virtual slides using image processing algorithms. This platform was programmed using C/C++ and MPI to carry out tissue-related measurements in a highly parallel fashion. Based on a *TMA-core Processor-core* correlation, a centralised load balancing approach was developed to utilise the processing power of all processor cores. Each TMA core is assigned for processing with a different processor core, whereas a master processor core is sacrificed to perform scheduling tasks. Other infrastructures were also developed to support the HPC platform, which include database and file access, as well as web-viewing through PathXL.
Our evaluations on texture feature calculation and biomarker quantification in a variety of TMAs showed significant speedups using <100 processor cores. The time for texture feature calculations was reduced to be 8 seconds per slide, comparing to 4 minutes using a standard computer. For the quantification of biomarkers, processing time was also greatly reduced from 19 minutes to 55 seconds per slide. With more than 9000 processor cores, >90 TMA slides could be processed simultaneously and instantly. This represents a significant advance in making TMA analysis a genuine high throughput technology and in enhancing tissue biomarker discovery programmes.

Parallel session 3: Cancer Genomics

PP13
MASSIVE GENOMIC COPY NUMBER MINING IN CANCER - ONCOGENOMICS MEETS SYSTEMS BIOLOGY

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Question: Genomic copy number abnormalities (CNAs) can be observed in the vast majority of malignanttissue samples. While recurring regional CNAs have been associated with specific gene targets,little is known about the interplay of extensive deregulation effects caused by the large and/ormultiple CNAs observed in many neoplasias. Descriptive meta-analyses of oncogenomicscreening data from Comparative Genomic Hybridization (CGH) experiments have been able toidentify imbalance patterns related to cancer entities and detected hithertounknown biological subsets. Although low resolution techniques have provided agood approximation of recurring genomic imbalance patterns, recent high resolution studies have pointed tothe additional involvement of small specific CNAs in oncogenesis. The true wealth ofCNA data can only be tapped by integrating comprehensive high-resolution, multi-disease CNA datasetswith systems biology approaches.
Methods: Previously, we have established the Progenetix database (www.progenetix.net) as the largestmanaged resource for chromosomal CGH data. Currently we are performing a massive oncogenomic datamining and annotation approach, based on the re-analysis of all publicly accessible whole genomecancer data sets. Parallel to this approach, we are analysing locus based CNA relationships andtheir relevance for neoplastic pathways, e.g. the NF-kB family in B-cell non-Hodgkin's lymphomas.
Results and conclusions: Our array CGH mining and re-annotation approach so far has allowed us to a) identify generalsources for oncogenomic screening data, b) catalog the vast range of platforms anddata types, and c) develop software tools and workflow

procedures for re-analyzing a majority ofdeposited oncogenomic array data. The processing of several thousand genomic profilesfrom a large range of platforms is well under way, and we will be able to present a general dataoverview as well as specific highlights during the meeting.

PP14
HIGH RESOLUTION ARRAY COMPARATIVE GENOMIC HYBRIDIZATION IN SPORADIC AND CELIAC DISEASE-RELATED SMALL BOWEL ADENOCARCINOMAS

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Purpose: The molecular pathogenesis of small intestinal adenocarcinomas (SBA) is not well understood. Understanding the molecular characteristics of SBA may lead to more effective patient treatment.
Materials and methods: Forty-eight SBA (33 non-celiac disease (CD)-related and 15 CD-related) were characterized for chromosomal aberrations by high resolution array comparative hybridization, microsatellite instability and *APC* promoter methylation and mutation status. Findings were compared to clinicopathological and survival data. Furthermore, molecular alterations were compared between CD-related and non-CD related SBA.
Results: DNA copy number changes were observed in 77% SBA. The most frequent DNA copy number changes found were gains on 5p15.33-5p12, 7p22.3-7q11.21, 7q21.2-7q21.3, 7q22.1-7q34, 7q36.1, 7q36.3, 8q11.21-8q24.3, 9q34.11-9q34.3, 13q11-13q34, 16p13.3, 16p11.2, 19q13.2 and 20p13-20q13.33 and losses on 4p13-4q35.2, 5q15-5q21.1 and 21p11.2-21q22.11. Seven highly amplified regions were identified on 6p21.1, 7q21.1, 8p23.1, 11p13, 16p11.2, 17q12-q21.1 and 19q13.2. CD-related and non CD-related SBA displayed similar chromosomal aberrations. Promoter hypermethylation of the *APC* gene was found in 48% non CD-related and 73% CD-related SBA. No nonsense mutations were found. 33% of non CD-related SBA showed microsatellite instability, whereas 67% of CD-related SBA were microsatellite instable.
Conclusions: Our study characterized chromosomal aberrations and amplifications involved in SBA. At the chromosomal level, CD-related and non CD-related SBA did not differ. A defect in the mismatch repair pathways seems to be more common in CD-related than in non CD-related SBA. In contrast to colon and gastric cancers, no *APC* nonsense mutations were found in SBA. However, APC promoter methylation seems to be a common event in CD-related SBA.

PP15
NO EVIDENCE FOR CLONAL OUTGROWTH OF STROMAL CELLS CARRYING SOMATIC GENETIC ALTERATIONS IN CERVICAL CARCINOMA

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Cancer-associated fibroblasts (CAFs) and leukocytes are major components of the tumour stroma cell fraction. CAFs have been recognized as important team-mates of cancer cells that play an important role in tumour development and progression. Studies in breast and colon cancer showed that tumour development is accompanied by clonal outgrowth of CAFs carrying somatic genetic alterations, like copy number alterations (CNAs) and loss of heterozygosity (LOH), elements associated with DNA aneuploidy.
Question: We made an attempt to identify the somatic genetic changes in stromal cells in a series of formalin-fixed, paraffin-embedded (FFPE) cervical cancers.
Methods: For this purpose multi-parameter DNA content flow cytometry, S-phase analysis, cell-sorting and genome-wide CNA and LOH analysis using a 6K SNP-array were used (Corver et al. Cancer Res. 2008). Archival tumour samples were selected based on high resolution DNA histograms (CV of the epithelial fraction (CV < 6) and relative low numbers of infiltrating leukocytes and high numbers of fibroblasts. For CNA and LOH analysis the vimentin-positive stromal cell fraction was compared with paired lymph

node or endometrial tissue from the same patient that was histologically normal.
Results: We show that the genome of the vimentin-positive stromal cell fraction of cervical cancers is not distinguishable from that of normal cells. This is supported by a low average S-phase (1.2 +/- 1.4%), absence of detectable DNA aneuploidy, CNAs and LOH and identical genotypes after SNP-analysis. These findings are in strong contrast to the sorted keratin-positive epithelial cell populations of which 75% show a DNA aneuploid fraction, a significantly higher total S-phase (14.6 +/- 8.1%), and high frequencies of CNAs and LOH in all keratin-positives. Hot-spots of LOH on chromosomes 3, 4 and 6 are confirmed by microsatellite analysis. The sorted vimentin-positive stromal cells only show retention of heterozygosity.
Conclusions: In contrast to previous studies but in concordance with the work of Allinen et al. (Cancer Cell 2004) and Qiu et al. (Nat. Genet. 2008) we conclude that the clonal outgrowth of cervical cancer-associated stromal cells carrying somatic genetic alterations is unlikely. Furthermore, we show that these stromal cell fractions can be used as a patient-specific internal (DNA diploid) reference for genome-wide CNA and LOH detection using low-density SNP-arrays. This will allow large retrospective genetic studies of archival tumour samples of which no paired normal tissue is available.

PP16
GENETIC PROFILE OF ADENOID CYSTIC CARCINOMA WITH TRANSFORMATION TO ADENOCARCINOMA AND UNDIFFERENTIATED CARCINOMA

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Transformation of adenoid cystic carcinoma (ACC) into poorly differentiated adenocarcinoma (AdACC) or undifferentiated carcinoma (UdACC) is a rare phenomenon and these tumors seem to be more aggressive than conventional solid ACC. However, ACC can also undergo transformation to adenocarcinomas which are not poorly differentiated and may perhaps not represent a more advanced or more aggressive tumor stage.
This study was aimed to compare genome-wide DNA copy number changes in ACC with transformation into adenocarcinomas with degrees of differentiation varying from intermediate to high and into UdACC versus conventional solid ACC, using high resolution microarray CGH. In addition, the genetic changes were correlated with clinical outcome and with Ki67 index and p53 expression, two immunohistochemical markers with prognostic value in ACC.
We studied 11 cases of ACC: 5 of the solid type, 5 with transformation into adenocarcinoma (3 moderately and 2 poorly differentiated) and 1 into undifferentiated carcinoma.
The transformed ACC group showed relatively simple genomic profiles, two cases carrying one single abnormality, whereas solid ACC demonstrated complex profiles. The expression of Ki67 and p53 was higher in the solid than in the transformed ACC group. Compared to the solid ACC, the transformed ACC group, except the UdACC case, showed a better overall and disease-free survival.
Our genetic, immunohistochemical and clinical data suggest that ACC with transformation into AdACC are less aggressive than solid ACC and the number of genetic abnormalities could be of prognostic value.

PP17
GENOMIC IMBALANCES IN TYPE I ENDOMETRIAL CARCINOMAS - COMPARISON OF DNA PLOIDY, KARYOTYPING AND COMPARATIVE GENOMIC HYBRIDIZATION

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DNA ploidy status is useful for prognostication of cancers. Generally, patients with DNA aneuploid lesions have poorer outcome than patients with diploid lesions. However, the detailed genetic changes underlying aneuploidy is not fully understood. We undertook this study to detect patterns of genetic

changes in DNA diploid, tetraploid and aneuploid tumors. Tumors from 51 patients with uterine endometrioid carcinoma were analyzed with respect to DNA ploidy status, karyotyping and copy number changes as detected by CGH.
As measured by image cytometry: 36 of 51 cases (71%) were DNA diploid, seven (14%) tetraploid, and eight (16%) aneuploid. Aberrations (gains and/or losses as measured by CGH) were found in 16 of 34 diploid tumors (47%). Ten (29%) had aberrations on chromosome 1, seven (21%) on chromosome 10, while in only one case (3%) aberrations were observed on chromosome 8. Only two of 34 (6%) cases showed above four copy number changes. Four of seven (57%) DNA tetraploid cases had CGH aberrations. Two of seven (29%) cases had above four copy number changes. In DNA aneuploid cases, six of eight (75%) had CGH aberrations. The aberrations were observed on chromosome 1 and/or chromosome 8 in five (63%) cases, while four (50%) cases had aberrations on chromosomes 7, 10, and X. Five of eight cases (63%) had above four copy number changes.
We observed a significant correlation between increasing DNA ploidy complexity and increasing number of copy alterations. DNA aneuploid carcinomas presented with a higher number of imbalances, with more chromosomes affected than tetraploid and diploid tumors. Aberrations on chromosome 8 might be a feature of DNA aneuploid, endometrioid endometrial carcinomas, since 63% of the aneuploid while only 3% of the diploid lesions, had aberrations on this chromosome.

PP18
***LKB1* PROMOTER AMPLIFICATION IN SPORADIC PEUTZ-JEGHERS POLYPS**

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Peutz-Jeghers syndrome (PJS) is a dominantly inherited disorder characterized by gastrointestinal hamartomatous polyposis, mucocutaneous melanin pigmentation and an increased risk of cancer at a relatively young age. Inactivation of tumor suppressor gene *LKB1* is the underlying germline defect in Peutz-Jeghers Syndrome patients.
Cases of sporadic PJ polyps have been reported, but their existence has been debated. It has been stated that these polyps are extremely rare and that the fact that no molecular data were found to support the PJS diagnosis is only due to failing techniques.
In this study we aimed to identify the molecular defect in 5 sporadic PJ patients. These patients are characterized by the presence of a distinct PJS polyp, most importantly the presence of arborizing smooth muscle, and the absence of a familial history and the distinct pigmentation.
Of these 5 patients we performed *LKB1* germline analysis and Multiplex Ligation-dependent Probe Amplification (MLPA) to detect exon deletions and amplifications. In one patient, the DNA quality was too poor to perform these analyses. In two of the remaining polyps no defects were found, in the final two polyps high level amplification of the *LKB1* promoter was identified.
Interestingly, we have identified *LKB1* promoter amplification in an additional 4 PJS patients in our PJS cohort. To study the effect of this amplification on gene expression, we performed a RT-PCR in two patients where frozen tissue was available, both normal and polyp tissue. A significant reduction of *LKB1* expression was found (20 fold reduction, p=0.0255) when PJS tissue was compared to a panel of 10 normal colon samples.
These results show that polyps that were based on histology sporadic PJ polyps, are indeed PJS polyps, and these patients should be monitored accordingly. Interestingly, amplification of the LKB1 promoter resulted in decreased protein expression. The exact mechanism leading to this reduction remains to be determined.

Parallel session 4: Cancer Epigenetics and Tumor Microenvironment

PP19
METHYLATION PROFILES IN ENDOMETRIAL CANCER OF DIFFERENT HISTOLOGICAL SUBTYPES

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Question: Promoter methylation is a gene- and cancer-type specific epigenetic event that plays an important role in tumour development. As endometrioid (EEC) and serous endometrial cancers (UPSC) exhibit different clinical, histological, and molecular genetic characteristics, we hypothesized that these differences may be reflected in epigenetic phenomena as well. Identification of a panel of methylation biomarkers could improve our understanding of carcinogenesis and could be helpful in a correct histological classification of these two subtypes that solely on the basis of morphology is not always easy.
Methods: Methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA) was used to assess the extent of promoter hypermethylation of 24 different tumour suppressor genes in 93 EEC and 26 UPSC. Methylation results were correlated with histology and survival.
Results: The number of methylated genes per tumour ranged from 0-8. The median cumulative methylation index (CMI) of all genes was significantly higher in EEC (177) than in UPSC (99) ($P < 0.001$). RASSF1A showed frequent methylation in both cancers. Promoter methylation of CDH13, GSTP1 and MLH1 was more frequently present in EEC, while CDKN2B and TP73 were more frequently methylated in UPSC. CDH13, MLH1, and TP73 were found to be the best predictors of EEC and UPSC histology. Almost 90% of EEC and 70% of UPSC could be predicted by this small panel. In EEC, methylation of MLH1 was associated with a shorter disease free ($p < 0.0001$) and overall survival ($p=0.005$). In a multivariate model, MLH1 methylation emerged as an additional prognostic factor to stage for DFS ($p=0.002$).
Conclusions: Promoter hypermethylation is more common in EEC than UPSC. Methylation of CDH13, MLH1 and TP73 predicted the correct tumour type best. Therefore, a panel of these methylation biomarkers could be useful to distinguish between the two histological subtypes of endometrial cancer. Further, methylation of MLH1 may have prognostic value in EEC.

PP20
METHYLATION TOLERANCE DUE TO O^6-METHYLGUANINE DNA METHYLTRANSFERASE (MGMT) FIELD DEFECT IN THE COLONIC MUCOSA: AN INITIATING STEP IN THE DEVELOPMENT OF MISMATCH REPAIR DEFICIENT COLORECTAL CANCERS

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Background and Aims: O^6-methylguanine-DNA methyltransferase (MGMT) removes methyl adducts from O^6-guanine. Known as methylation tolerance, selection for mismatch repair (MMR)-deficient cells that are unable to initiate lethal processing of O^6-methylguanine-induced mismatches in DNA is observed *in vitro* as a consequence of MGMT deficiency. We therefore hypothesized that MGMT field defect may constitute a pre-neoplastic event for the development of MMR-deficient tumours displaying microsatellite instability (MSI).
Methods: MGMT status was investigated by immunohistochemistry and the methylation status of the gene promoter by PCR in neoplastic, adjacent and distant mucosal tissues of patients with MSI or non-MSI (MSS) CRC. The cancers were familial (42 MSI, 13 MSS) or sporadic (40 MSI, 49 MSS) in origin, or arose in the context of inflammatory bowel disease (IBD; 13 MSI, 36 MSS). Colonic mucosa from patients with diverticulitis ($N = 20$) or IBD ($N = 39$ in 27 patients) without cancer served as controls.

Results: Loss of MGMT expression was more frequent in MSI than MSS CRC (P = 0.047). In comparison to MSS tumours, MSI CRC occurred more frequently adjacent to patches of mucosa that lacked MGMT expression (P = 0.002). Overall, loss of MGMT expression was associated with MGMT gene promoter methylation (P = 0.03).
Conclusion: MGMT field defects are more frequently associated with MSI than MSS CRC. These findings indicate that methylation tolerance may be a crucial initiating step prior to MMR deficiency in the development of MSI CRC in both familial and sporadic settings.

PP21
RASSF1A METHYLATION ENHANCES K-RAS MUTATIONS IN BLOCKING EGFR PATHWAY

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Detection of K-ras and BRAF gene mutations is mandatory to set a therapy with anti-EGFR antibodies (cetuximab and panitumumab) in metastatic colorectal carcinoma. We hypothesized the potential role of tumor suppressor methylation in inhibiting EGFR signaling cascade thus blocking anti-EGFR therapeutic effect and in facilitating metastatic progression.
Primary tumor and liver metastatic tissues of 50 patients affected by mCRC have been characterized for promoter methylation of p16, RASSF1A and RAR beta suppressor genes by Quantitative Methylation Specific PCR (QMSP). Twenty patients also had metastatic lymph node tissues analyzed. Moreover, all cases were analyzed for K-RAS (codons 12 and 13) and BRAF (codon 600) mutations.
RARbeta, RASSF1A and p16 genes resulted methylated in 82%, 34.7% and 25.3% of primary tumors respectively. Interestingly, RASSF1A and p16 resulted significantly more frequently methylated in liver metastasis than in primary site (p=0.015 and p<0.01, respectively). On the contrary, RAR beta methylation percentage was significantly lower in distant metastasis with respect to primary site (p=1.2x10-6). Methylation percentage of all 3 genes in lymph node is always lower than in the other two sites (RARb: p=0.005).
As regards methylation content, RASSF1A methylation status is significantly higher in liver metastasis with respect to primary tumor (Wilcoxcon p=0.000) underlying the role of this gene in liver metastatic progression.
In our series K-RAS resulted mutated in 39% and BRAF in 9% of cases. Both methylation frequency and level seem to be unrelated to gene mutation, apart for RASSF1A (20% of RASSF1A methylated cases are also K-RAS mutated, p=0.05). RASSF1A inactivation, through promoter hypermethylation, is a frequent alteration observed in sporadic colorectal cancer. Accordingly to what reported in the previous literature, RASSF1A promoter hypermethylation occurs dominantly in tumors with KRAS wild type, even if the quantity of methylation is lower. On the contrary, K-RAS mutations, particularly G12D and G13D, were associated to high methylation level of RASSF1A gene stressing the function of this gene in strengthening the apoptotic role of K-RAS in primary site.
These evidences support the role of oncosuppressor methylation in both colon tumorigenesis and progression suggesting that the epigenetic events have to be taken into account when biological therapies have to be set. Moreover we verified the role of RASSF1A in the enhancement of blocking EGFR pathway.

PP22
COMPARISON OF DIFFERENT PROMOTER METHYLATION ASSAYS IN BREAST CANCER

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Gene promoter hypermethylation is increasingly being recognized as a promising biomarker for cancer detection. Currently, different quantitative and non-quantitative techniques are used to measure gene methylation in clinical specimens with varying results. Here we performed a side-by-side comparison of three commonly used methods to determine to which extent these discrepancies can be explained by the differences between the assays.
We used Methylation-Specific PCR (MSP), Quantatitive Multiplex Methylation Specific PCR (QM-MSP) and Methylation Specific-Multiplex Ligation-dependent Probe Amplification (MS-MLPA)

to compare the methylation of 4 genes commonly methylated in breast cancer (*CCND2, SCGB3A1, RARB* and *RASSF1*) on DNA isolated from 40 invasive breast carcinomas. The influence of tissue fixation, location of the target sequence and the amount of DNA on the performance of these assays was studied.
MSP and QM-MSP provided highly discrepant results. 20% of tumors that showed no methylated band in MSP gave >10% methylation in QM-MSP, and 10% of these samples even showed > 50% methylation. In contrast, there was a strong correlation (Pearson correlation coefficient 0.73) between methylation values obtained through QM-MSP and MS-MLPA targeting the same sequence in DNA isolated from paraffin embedded tissue. This correlation declined with non-overlapping target sequences (0.56) or when fresh frozen tissue (0.60) was used. In titration experiments, MSP and MS-MLPA still performed robust with 10 ng of DNA, while QM-MSP was at least ten-fold more sensitive.
In conclusion, despite the difference in molecular basis, the quantitative methylation assays QM-MSP and MS-MLPA showed moderate to strong correlations. In contrast, there was a poor concordance between either of these quantitative techniques and non-quantitative MSP. For biological samples with low amounts of DNA, QM-MSP is the method of choice.

PP23
CELLULAR AND MOLECULAR MECHANISMS OF THE VASCULAR DISRUPTING EFFECT OF THE ANTI-CANCER DRUG 5,6-DIMETHYLXANTHENONE-4-ACETIC ACID (DMXAA)

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5, 6-dimethylxanthenone-4-acetic acid (DMXAA; ASA404; vadimezan) is an effective anti-cancer agent developed at the Auckland Cancer Society Research Center and is currently undergoing phase III clinical trials (Novartis). Previous studies have shown that DMXAA selectively disrupts tumour vasculature as part of its anti-cancer action. Question: Our study has focused on what are the cellular and molecular changes that DMXAA induces in endothelial cells. Methods: Human umbilical vein endothelial cells (HUVECs) were cultured on the extracellular matrix material Matrigel to form capillary-like networks or on gelatin coating for scratch assays. Time lapse microphotography were taken to record the movements of the cells. Immunofluorescence stainings were carried out to visualize cytoskeletal fibers. Mass spectrometry was carried out to measure the concentrations of ceramides. Results: The network formation process was accelerated and combined with changes in cell shape by treatment either with 30 to 300μM DMXAA or with 50ng/ml vascular endothelial growth factor (VEGF), as measured by time-lapse microphotography. Immunostaining of HUVECs showed both DMXAA and VEGF induced actin stress fiber formation, consistent with an involvement in this increased migration. The mobility of HUVECs growing on gelatin did not show significant change by either DMXAA or VEGF treatment, as measured by scratch assays, indicating that Matrigel contains components that facilitate the effect of the drugs. Mass spectrometry showed that C16 and C24 ceramides increase by 17 hours of DMXAA treatment in HUVEC networks. Conclusion: DMXAA disrupted cytoskeleton of endothelial cells and thus influenced their mobility. These effects were similar to the effects caused by VEGF. DMXAA possibly acts via a pathway involving ceramide synthesis, p38 kinase, and the consequent actin stress fiber formation. The role of P38 kinase is under investment currently by using P38 kinase inhibitors.

PP24
TUMOR CELLS MODULATE PHENOTYPE AND FUNCTION OF HUMAN BONE MARROW-DERIVED MESENCHYMAL STROMAL CELLS IN A NON-CONTACT DEPENDENT MANNER

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Introduction: Many solid tumors, including breast and prostate cancers, have a predisposition to metastasize to the bone marrow where they might negatively influence hematopoiesis. Mesenchymal stem cells (MSCs) are the progenitors for all stromal cells in the bone marrow and produce cytokines and chemokines to support hematopoietic stem cells (HSCs). In this study we investigated a possible influence of prostate

and breast carcinoma cells on MSCs in a coculture model.
Methods: Human MSCs (derived from the bone marrow of healthy volunteers and the immortalised MSC line SCP-1) were cocultured with tumor cell lines (MCF-7 and PC-3) or incubated with conditioned medium of these cells. The proliferation potential was detected by BrdU staining or by MTT assay. Flowcytometric analysis included MSC, HSC as well as tumor markers (CD34, CD44, CD45, CD73, CD90, CD105, CD146, CD166, CXCR4). SDF-1 mRNA levels of MSCs were quantified by real-time PCR and the levels of secreted protein were measured using an ELISA kit.
Results: MSC/MCF-7 and MSC/PC-3 cocultures showed a lower MSC proliferation activity in comparison to MSC control cultures. SCP-1 proliferation was decreased to 52.5% by MCF-7 conditioned medium and to 63.4% by PC-3 conditioned medium which suggests that the effect on proliferation is cell contact independent. Furthermore, incubation with tumor cell conditioned medium caused a decrease in the positive fraction and mean fluorescence intensity of CD105 and CD146 on MSCs. SDF-1 concentrations in coculture supernatants decreased in a time-dependent manner and the effect was stronger in cocultures with MCF-7 cells (on average MSCs 717 pg/ml; MSC/MCF-7 145 pg/ml; MSC/PC-3 507 pg/ml). Incubation with tumor cell conditioned medium caused a significant decrease of SDF-1 secretion. Interestingly, this effect could be reversed after a medium change to normal DMEM. In accordance with the SDF-1 protein levels the mRNA levels were downregulated in MSCs incubated with MCF-7 conditioned medium.
Conclusion: The phenotypic and functional changes induced in MSCs during cocultures with tumor cells or conditioned medium, respectively, suggest a negative impact on the support of MSC towards hematopoietic stem cells. The coculture model chosen allows to mimic the competition between tumor cells and HSC for the stromal niche compartment in-vitro.

Parallel session 5: Metastasis Formation

PP25
COMPUTER SIMULATION OF THE METASTATIC CASCADE

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The rate-limiting step of the metastatic cascade is still subject of intense discussion and even established theoretical models differ considerably in several basic details. Mathematical models of metastasis formation would be desirable since they have the advantage that their predictions can be examined and verified quantitatively. Yet only one mathematical model is known to us that describes the entire process of (local) metastasis, which is, however, too inflexible to include distant metastasis formation or to describe the effects of treatments of the metastatic process.
Therefore, a novel computer model was developed that is able to describe quantitatively the processes underlying the metastatic cascade. The computer model is based on a discrete event simulation procedure. The growth of the primary tumor is described via analytical functions, while a rate function models the intravasation events of the primary tumor and its metastases. Events describe the behavior of the emitted malignant cells until the formation of new metastases. The results of the computer simulations are in quantitative agreement with clinical data determined from a patient with hepatocellular carcinoma in the liver. The model provides a more detailed view on the process than a conventional mathematical model. Different growth functions can be applied for the primary tumor and metastases or even different types of metastases. In particular, the implications of interventions on metastasis formation can be calculated, which is difficult to describe with mathematical models proposed so far.

PP26
CYTOMIC ANALYSIS OF CHEMOKINE RECEPTORS AND THEIR LIGANDS IN HUMAN MELANOMA CELL LINES

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Chemokines have been implicated in tumor progression and metastasis. In melanoma, chemokine receptors have been implicated in organ selective metastasis by regulating processes such as chemoattraction, adhesion and survival. In particular, CCR7 has been implicated in lymph node metastasis, CXCR4 in pulmonary metastasis, and CCR10 in skin metastasis, using a mouse model of melanoma. In human melanoma, it is known that primary and metastatic cancer cells express several chemokines receptors and that their chemokine ligands are highly expressed in metastasis sites.
In this study, we have analyzed the systems formed by the chemokine receptors CXCR3, CXCR4, CXCR7, CCR7, and CCR10 and their ligands in human melanoma cell lines, using flow cytometry. We have characterized 13 melanoma cell lines, 5 established from primary tumors and 8 established from metastasis from different tissues. Also, we extracted RNA from all cell lines for quantification of gene expression of the chemokines receptors and ligands.
Our results show that the melanoma cell lines do not express or express in a lower degree the chemokine receptors on their cell surface, while the control HUT78 cell line shows surface expression of CXCR3, CXCR4, CCR7 and CCR10. However, we have found the interesting fact, not described before, that melanoma cell lines show intracellular expression of all the forementioned receptors and most of their respective ligands. Also, the pattern of intracellular expression of chemokines is very similar between the different cell lines. The control HUT78 cell line expresses all the receptors but does not express or expresses in a lower degree ligands for CXCR3, CXCR4, CCR7 and CCR10.
These results suggest that the autocrine production of chemokines by the melanoma cell lines may block the expression of the receptors on the cell surface. The inhibition of the chemokine ligands production with interference RNA, and co-localization studies using confocal microscopy, will be done to further clarify this hypothesis. Also, a more in-depth comprehension of these systems formed by the chemokine receptors and their ligands will be provided by the quantitative studies of gene expression using real time PCR.

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PP27
E2F1 DETERMINES MELANOMA PROGRESSION AND METASTASIS VIA EGF RECEPTOR SIGNALING

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Question: Metastases are responsible for cancer deaths, but the molecular alterations leading to tumor progression are unclear. Overexpression of the E2F1 transcription factor is common in high-grade tumors that are associated with poor patient survival. Methods: To investigate the association of enhanced E2F1 activity with aggressive phenotype, we performed a gene-specific silencing approach in a metastatic melanoma model. *Results:* Knockdown of endogenous E2F1 via E2F1 small hairpin RNA (shRNA) expression increased E-cadherin expression of metastatic SK-Mel-147 melanoma cells and reduced their invasive potential but not their proliferative activity. Although growth rates of SK-Mel-147 and SK-Mel-103 xenograft tumors expressing E2F1 shRNA or control shRNA were similar, mice implanted with cells expressing E2F1 shRNA had a smaller area of metastases per lung than control mice (n = 3 mice per group; 5% vs 46%, difference = 41%, 95% confidence interval = 15% to 67%; P = .01; one-way analysis of variance). We identified epidermal growth factor receptor (EGFR) as a direct target of E2F1 and demonstrated that inhibition of receptor signaling abrogates E2F1-induced invasiveness.
Conclusions: The data emphasize the importance of the E2F1 - EGFR interaction as a driving force in

melanoma progression that may serve as a paradigm for E2F1-induced metastasis in other human cancers.

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PP28
MOLECULAR CHARACTERIZATION OF THE TUMOR SUPPRESSOR ACTIVITY OF PLAKOGLOBIN (G-CATENIN) AND ITS ROLE IN REGULATION OF GROWTH CONTROL AND METASTATIC PATHWAYS

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Plakoglobin (Pg, g-catenin) is a homolog of b-catenin with similar dual adhesive and signaling functions. The adhesive function of these proteins is mediated by interactions with cadherins, whereas association with various intracellular partners regulates their signaling activity. In this respect, b-catenin has known oncogenic activity via its role in the Wnt signaling pathway, while Pg acts as a tumor/metastasis suppressor by unknown mechanism(s).
We developed an experimental model system using human squamous and mammary carcinoma cell lines that lack Pg, have transformed morphologies and exhibit different degrees of invasiveness to investigate the underlying mechanism(s) of Pg's tumor suppressor activities. These cell lines were used to develop transfectants with different levels of expression and subcellular localization of Pg to assess its role at the membrane, in the cytoplasm and in the nucleus. The transfectants were then characterized by a multifaceted approach including proteomics and transcriptome analyses.
The results of these analyses have provided strong evidence that Pg can function as a growth regulator and tumor/metastasis suppressor in both the cytoplasm and nucleus by altering the expression and stability of growth regulatory molecules. Non-metastatic 23 (Nm23-H1/H2) and nucleophosmin (NPM), a nuclear/nucleolar protein with dual oncogenic and tumor suppressor activities were among the identified molecules and were further characterized. RT-PCR and immunoblotting showed increased Nm23 and NPM transcripts and protein levels upon Pg expression. Preliminary chromatin immunoprecipitation showed that Pg binds to the promoter of Nm23-H1and NPM2. Coimmunoprecipitation and confocal microscopy revealed that Pg interacted with Nm23 and led to its membrane localization in a complex with cadherins and a-catenin. Similar studies verified Pg-NPM interactions and their nuclear/nucleolar colocalization in normal epithelial cells and its absence in invasive carcinoma cell lines. Further, Pg's shuttling in and out of the nucleus was necessary for NPM localization.
Together, these studies suggest that Pg may function as a tumor/metastasis suppressor by altering the expression and/or subcellular localization of various molecules involved in tumorigenesis and metastasis.

PP29
IDENTIFICATION OF GENOMIC ALTERATIONS ASSOCIATED WITH METASTASIS AND POOR PROGNOSIS IN CLEAR CELL RENAL CELL CARCINOMA

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Introduction and objectives: Clear cell renal cell carcinoma (ccRCC) is the most frequently encountered renal malignancy with a high metastatic potential and the prognosis of patients in this subgroup is very poor. So the aim of our study was to identify regions of DNA copy number changes significantly associated with metastasis and clinical outcome of patients with ccRCC.
Materials and methods: We conducted genome-wide copy number profiles in 56 primary ccRCC including 32 metastasized and 24 non-metastasized tumours by array CGH with a median resolution of 1-1,5MB. The median follow up of the patients was 60 months.
Results: We identified 6 recurrent chromosomal aberrations: gains of 1q21.3, 12q13.11, 12q13.2 and 20q11.21q13.2 and losses of 8p11.23p12 and 9p21.3p24.1, which were significantly associated with metastasis occurrence. In multivariate analysis including these aberrations gains of 1q21.3 and of 20q11.21q13.2 as well as loss of 9p21.3p24.1 attained as independent predictors for metastasis in our cohort with 81.5% sensitivity and 95.8% specificity. Kaplan-Meier survival analysis showed that gains on chromosomes 7, 12, 16 and 20q and losses on chromosome 9 have significant correlation with disease specific survival. Multivariate Cox-regression

analysis retained gains of 7q36.3 and of 20q11.21q13.2 and a loss of 9p21.3p24.1 as independent prognostic factors for outcome of patients.
Conclusions: Our data suggest that certain common copy number alterations in ccRCC could serve as independent predictors for metastasis occurrence and cancer specific survival in patients with ccRCC.

PP30
IDENTIFICATION OF A microRNA SIGNATURE ASSOCIATED WITH METASTASIS IN CLEAR CELL RENAL CELL CARCINOMA

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Background: Recent studies have shown that microRNAs (miRNAs) play an important role as regulators of gene expression in tumourigenesis. But their specific expression levels in clear cell renal cell carcinoma (ccRCC) and the influence on metastasis is almost unknown. We aimed at identifying specific miRNA expression patterns characterising ccRCC. Furthermore, it is the intention of this study to detect specific miRNAs regulating metastasis.
Material and Methods: MiRNAs were isolated and enriched from a total of 24 tissue samples, containing 10 non-metastatic tumours, 4 tumours with metastasis after 3 years and 4 tumours with primary metastasis as well as 6 normal non-tumour tissues obtained distant from the tumour. MiRNA expression analysis was performed by using microarrays and validated by using qRT-PCR
Results: We detected clear differences concerning miRNA expression between ccRCC and their normal non-malignant counterparts. Furthermore, we found highly significant differences in miRNA expression between metastatic and non-metastatic ccRCC. Thus, we identified a miRNA signature of 33 differently expressed miRNAs in metastatic ccRCC. In addition, we found significant correlations between expression levels of specific miRNAs (for example miR-10a, miR-24, miR-26a and let-7c) and survival of the patients.
Conclusion: Our findings indicate that specific miRNAs are involved in cancerogenesis and have an impact on the metastasis of the ccRCC. Furthermore, we identified specific miRNAs characterising very aggressive tumours with early metastasis. In addition we determined candidate markers associated with survival of the patients. Thus, detailed analysis of the primary tumour could be a helpful tool for prediction of individual prognosis and for therapy selection.

Parallel session 6: Tumor Biology and Response to Therapy

PP31
REDUCING CARCINOGENIC RISK IN A CELL CULTURE MODEL FOR CHEMO-ENDOCRINE RESISTANT BREAST COMEDO DUCTAL CARCINOMA IN SITU

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Study Rationale: In the multi-step process of human mammary carcinogenesis comedo ductal carcinoma in situ (DCIS) represents a pre-invasive lesion at risk for developing chemo-endocrine resistant breast cancer. This lesion is characterized by the presence of ER-/HER-2+ epithelial cells. Reliable human tissue derived epithelial cell culture model that expresses clinically relevant predisposing genetic defect and exhibits quantifiable risk for carcinogenesis should reduce extrapolation of the data for their clinical translatability. Experimental Model: The 184-B5/HER cell line that stably expresses HER-2 oncogene in ER-/PR- human mammary epithelial cells represented a model for comedo DCIS.
Study Outcome: Relative to the parental 184-B5 cells the HER-2 oncogene expressing 184-B5/HER cells exhibited 51.6% decrease in the population doubling time, 55.6% decrease in G1: S+G2/M ratio, 33.3% increase in saturation density and 11 fold increase in the S+G2/M: subG0 ratio, indicating loss of homeostatic growth control. The 184-B5/HER cells also exhibited anchorage independent colony formation in vitro and tumorigenecity in vivo, indicating enhanced carcinogenic risk. Treatment of aberrantly proliferative pre-neoplastic 184-B5/HER cells with select mechanistically distinct natural phyto-chemicals such as Rosemary terpenoids, tea polyphenols, soy isoflavone and grape phyto-allexin induced cytostatic growth arrest, altered cell cycle progression and enhanced cellular apoptosis, thus

reestablishing homeostatic growth control. Treatment with these natural phytochemicals also inhibited anchorage independent colonies, thereby reducing carcinogenic risk.
Study Conclusion: These data characterize a novel cell culture model for clinical comedo DCIS and validate a mechanism based approach to screen and prioritize new efficacious lead compounds for prevention/therapy of ER-/PR-/HER-2+ clinical breast cancer.

PP32
THE MECHANISM OF ACTION OF ZOLEDRONIC ACID IN BREAST CANCER

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Question: Zoledronic acid (ZA) has been recently shown to increase the progression free-survival of estrogen receptor (ER)-positive breast cancer patients by reducing both loco-regional and distant metastases. However, ZA very rapidly concentrates in the bone following intravenous administration. Recent reports have shown that bone-marrow-derived mesenchymal stem cell (MSCs) are recruited to the stroma of developing tumors, where they increase the metastatic potential of breast cancer cells by secreting the chemokine RANTES (CCL5) that sustains breast cancer motility and invasion. Therefore, we investigated the effects of ZA on the ability of primary MSCs to secrete factors that might be involved in breast cancer progression.
Methods: The antiproliferative effects of ZA on human primary MSCs were evaluated with an anchorage-dependent growth assay. The effects of ZA on the secretion of RANTES, IL-6 and angiogenic factors were assessed by using the Luminex-based Bio-Plex Suspension Array. The ability of breast cancer cells to migrate through a fibronectin-coated membrane was evaluated by using a commercially available assay.
Results: We found that treatment with ZA produced marginal effects on the growth of human primary MSCs, with an approximately 25% growth inhibition following treatment with 20 μM ZA for 48 hours. In contrast, conditioned medium from ZA-treated MSCs showed a reduced ability to promote the migration of ER-positive MCF-7 breast cancer cells through a fibronectin-coated membrane as compared with conditioned medium from untreated cells. In co-culture assays, treatment with ZA reduced the ability of MSCs to sustain the growth of breast cancer cells. We found that ZA almost completely suppressed the ability of MSCs to secrete RANTES. The effect of ZA on RANTES was quite specific, since marginal inhibition of the secretion of different angiogenic growth factors, such as VEGF, IL-8 and bFGF, was observed. ZA also significantly reduced the secretion by MSCs of IL-6 that has been previously demonstrated to act as a potent paracrine growth factor for human breast cancer cells.
Conclusions: Taken together, these data suggest that ZA might exert its antitumor activity in the bone marrow microenvironment by reducing the ability of MSCs to secrete factors involved in breast cancer progression.

PP33
REVERSIBLE POLYPLOIDY OF TUMOUR CELLS AS A SURVIVAL RESPONSE TO GENOTOXIC TREATMENTS

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Objectives: The phenomenon of reversible polyploidy in response to genotoxic treatments has been reported in tumour cells by several independent groups, although is poorly understood. In this paper, we focused on the study of the underlying cellular mechanisms.
Methods: Reversible polyploidy was induced in human lymphoma, ovarian teratocarcinoma, and cervical carcinoma cell lines by gamma-irradiation, etoposide and paclitaxel. Cellular changes were followed in an extended time-course by DNA cytometry, immunofluorescence, FISH and electron microscopy.
Results: The main steps of reversible polyploidy are as follows: (1) Polyploidisation to 8-32C by abortive mitoses followed by re-replication. (2) DNA recombination and repair, with participation of meiotic proteins REC8 and DMC1. (3) Segregation of chromosomes from multi-genomic tumour cell nuclei by mitotic spindles in a bi-polar or multi-polar fashion followed by meiotic-type chromosome reduction. (4) Fusion between daughter sub-nuclei of neighbour bi-polar anaphases followed by AURORA B-kinase aided cytotomy (usually incomplete) of fused products in multi-nuclear cells. (5) Extensive macroautophagy combined with chromatin diminution, in parallel to division activities. Diminution involves sorting of acentrics. (6) Autolysosomes release daughter sub-cells from the polyploid mother enabling final dissipation of self-renewed (NANOG-positive, p16-negative) sub-cells. The released sub-cells immediately start mitotic

divisions. At least 75% of metaphases in the cells marked for the remnants of autophagic vacuoles (in etoposide-treated WI-L2-NS cell line, day 14) contain 4C DNA. *Conclusion:* The steps of reversible polyploidy bear the features of a life-cycle, rejuvenate tumour cells and restore diploidy.

PP34
THE IMPACT OF CHROMATIN DENSITY ON RADIORESISTANCE OF HUMAN TUMOR CELLS

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Three-dimensional (3D) cell culture models provide a more physiological approach to the analysis of ex vivo cell behaviour upon exposure to external stress. As compared to a flat cell morphology found under two dimensions, 3D growth exhibits a round cell shape. Mediated by direct interactions between extracellular matrix (ECM), integrins, actin cytoskeleton and nuclear membrane, the physical forces engaged are dramatically different between 2D and 3D conditions. Emerging findings suggest these differences to substantially impact on chromatin organization and gene expression, which in turn contributes to the regulation of cell survival and DNA repair. In malignant tumors, these actions may promote radio- and chemoresistance. In 3D, a higher portion of condensed chromatin is reflected by elevated heterochromatin protein (HP) 1-alpha and decreased histone H3 acetylation. To evaluate the role of chromatin condensation on radioresistance, h*uman* A549 lung cancer cells grown in 3D cell cultures, as xenograft tumors and as monolayer were compared. Clonogenic radiation survival (0-6 Gy), number of radiogenic residual DNA double-strand breaks (rDSB, gammaH2AX-S139 foci assay), chromosomal aberrations (G0 assay, Spectal Karyotyping) and protein expression (acetyl-H3, HP1-alpha) were examined. To analyze colocalization of gammaH2AX-S139 and HP1-alpha to distinguish eu- from heterochromatic foci, cells were stably transfected with EGFP-HP1-alpha. The data show that increased levels of heterochromatin in 3D cell cultures result in increased radiation survival and in reduced numbers of rDSBs and lethal chromosome aberrations. Intriguingly, eu- to heterochromatin associated DSBs were equally distributed in irradiated 3D cell cultures and xenograft tumors while irradiated monolayer cultures showed more eu- than heterochromatin DSBs. Taken together, these data show an interrelation between cell morphology and cellular radioresistance essentially based on chromatin organization. Due to the fact that the accurate repair of radiogenic DNA damage occurs in the context of chromatin, these results might be highly relevant for better understanding the underlying molecular mechanisms by which chromatin structure influences the processing of radiation-induced DNA lesions.

PP35
***Msh2* STATUS MEDIATES AZATHIOPRINE-INDUCED CARCINOGENESIS IN MICE**

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Azathioprine is a thiopurine prodrug used extensively as an immunosuppressant in organ transplantation, autoimmune and inflammatory diseases. Thiopurine exposure has been observed to result in the selection of mismatch repair (MMR)-deficient cell clones *in vitro*. Moreover, it has been suggested that thiopurine drugs might constitute a risk factor for the emergence of human neoplasms displaying microsatellite instability (MSI), an oncogenic process that occurs due to MMR deficiency. To address this question, we administered azathioprine to mice that were wild type or inactivated in one or two copies of the *Msh2* MMR gene. $Msh2^{WT}$ and $Msh2^{+/-}$ control mice remained asymptomatic, however their azathioprine-treated counterparts developed lymphomas that were respectively MMR-proficient or displayed MSI due to somatic inactivation of the remaining functional *Msh2* allele. In contrast, azathioprine delayed the onset of MSI lymphomas in $Msh2^{-/-}$ mice. Our findings show this drug can mediate oncogenesis in mice according to their *Msh2* status. They notably highlight that this drug is able to trigger carcinogenesis through an MSI-driven process that may act synergistically with other oncogenic mechanisms related to its immunosuppressive effects. These observations could have important clinical implications for patients receiving azathioprine therapy.

PP36
FUNCTIONAL ANALYSIS OF pRb2/p130 AND Cdk9: IMPLICATIONS IN CANCER, VIRAL PATHOGENESIS, DRUG DEVELOPMENT AND GENE THERAPY

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The putative pRb2/p130 tumor suppressor gene and cyclin-dependent kinase 9 (Cdk9) are two key battlegrounds in my research center. These molecules were identified and characterized in my laboratory in the early 1990s and they both became trend-setting topics in the areas of cancer research and cell biology.
The retinoblastoma (Rb) gene family comprises three members: the Rb tumor suppressor gene pRb/p105, pRb/p107 and pRb2/p130, which are also known as pocket proteins, as their structure resemble the shape of a pocket. The three components of the Rb family share similar structural identities, arrest cell cycle progression in G1 phase, their phosphorylation patterns are cell cycle dependent and the inhibition of cell proliferation takes place in a cell-type-specific pattern, which indicates that the biological functions of the pocket proteins are not completely redundant.
The inactivation of tumor suppressor genes is quasi-obligatory for the establishment of a malignant cell phenotype. Loss of tumor suppressor gene expression may occur via deletion mechanisms of particular chromosomal areas, epigenetic and/or genetic mutations, a combination of the two aforementioned events and because of certain viral factors. For instance, the human papilloma viral (HPV) E7 protein binds and neutralizes Rb proteins. The same occurs with polyoma viruses large T antigens. The large T antigen of the JCV binds pRb2/p130 and causes the release of E2F, which, in turn, exerts its effects on cell cycle regulation.
Human pRb2/p130 gene is encoded in chromosome 16q12.2. A number of human tumor-derived cell lines exhibit deletions and /or mutations in this chromosomal area, such as hepatic, breast, prostate and ovarian cancers. Interestingly, retroviral-mediated gene transfer of human pRb2/p130 could inhibit human ling cancer cell proliferation *in vitro* and in xeno-transplanted nude mice.
In addition to gene-based approaches for the expression of human pRb2/p130 in cancer cells, we developed a pRb2/p130-derived peptide termed Spa310, which is a 39 amino acids proteins spanning part of the spacer region. Interestingly Spa310 caused *in vitro* inhibition of cdk2 activity, substantial arrest of proliferation in mouse NIH/3T3 fibroblasts and inhibition of human lung tumor growth *in vitro* and in xeno-transplanted mice.
In conclusion: gene-based interventions and use of small peptides derived from pRb2/p130 have in common the elimination of malignant cells by imparting cell cycle arrest through inhibition of cdk2 activity. Studies are currently in progress to understand the mechanism of Spa 310 entry into cells and tumor growth suppression in xeno-transplanted mice.

Cdk9
Unlike other Cdks, Cdk9 is not directly involved in cell cycle regulation, but promotes RNA pol II-mediated transcription to sustain genetic programs related to cell proliferation, growth, survival and differentiation. Cdk9 cyclin partners are cyclin T1, cyclin T2a, cyclin T2b and cyclin K. The heterodimer "Cdk9/cyclin partner" is stable and constitutes a major component of the positive transcription elongation factor b (P-TEFb), which stabilizes the elongation of RNA pol II-driven RNA transcripts. The complex biology of Cdk9 can be summarized as follows:
1) The Cdk9-related pathway is a key component in the regulation of mammalian gene expression, which controls several cellular processes, such as cell growth, proliferation, survival and differentiation, depending on the cell context.
2) Cdk9/cyclin T1 are involved in the replication cycle of HIV-1. HIV-2, HTLV-1, EBV and HSV-1.
3) Deregulations in the Cdk9-related pathway are associated with various pathological conditions, which include cancer and cardiac hypertrophy.
4) The study of the mechanisms that lead to an aberrant Cdk9-related pathway is essential for the development of novel kinase inhibitors for the treatment of cancer, AIDS and cardiac hypertrophy and might shed useful insights into the study of the pathogenesis and progression of these maladies.

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