416 Characterization and quantification of macrophages in colorectal cancer by an automated cell system

R. Rogojanu[1], I. Mesteri[2], I. Ellinger[1], T. Talhammer[1], E. Kallay[1], A. Heindl[1], A. Seewald[3], G. Bises[1]. [1]*Medical University of Vienna, Department of Pathophysiology, Vienna, Austria,* [2]*Medical University of Vienna, Department of Pathology, Vienna, Austria,* [3]*Seewald, Solutions, Vienna, Austria*

Background: Communication between tumour and its surroundings (the tumour microenvironment) is of utmost importance in determining the faith of the tumour. Both pro- and anti-tumour interactions have been described.
In order to understand the role of tumour-associated macrophages in malignant progression we investigated the distribution of total macrophages CD68+ and M2 anti-inflammatory activated macrophages CD163+ in colorectal cancer and adjacent mucosa using a fully automated microscope-based cell analysis system that performs in-situ analysis of multiple markers in complex tissues like tumour specimens.
Material and Methods: Immunofluorescent staining was performed in paraffin sections from 8 G2 colorectal tumours. 4 patients have already developed liver metastasis. Sections from mucosa adjacent to tumour tissue as well as 3 healthy mucosae were examined. Large tissue sections of around 2 cm^2 were scanned using TissueFAXS Cytometer and analysed by TissueQuest analysis software (TissueGnostics GmbH, Austria).
Results: The analysis of the adjacent mucosa revealed a heterogeneous increase in patients regarding local density of CD68+ and CD163+ compared with healthy mucosa. Numbers of CD68+ cells at the tumour front increased twofold in patients without liver metastasis and threefold in patients with liver metastasis. The tumour centre of patients without liver metastasis showed the lowest amount of CD68+ infiltrating cells. Tumour centre of patients with liver metastasis revealed a 4 times increase of CD68+ cell density when compared with patients without liver metastasis. CD163+ cells were present in all tumour areas with high variability, with higher density in patients without liver metastasis.
Of special interest is an observation regarding the presence of mono- or multinucleated CD163−/CD68+ giant cells in tumour and/or adjacent mucosa of all patients without liver metastasis. Only 2 adjacent mucosae from patients with liver metastasis showed macrophages with giant cell phenotype.
Conclusions: Our data suggests that CD68+ giant cells might be a marker for favourable prognosis in colorectal cancer. Further studies are needed to characterize their function.

417 A c-Myc induced gene expression signature in human germinal center B cells predicts subtypes of aggressive non-Hodgkin Lymphoma

A. Schrader[1], S. Bentink[2], R. Spang[3], D. Lenze[4], M. Hummel[4], M. Kuo[5], P. Murray[6], L. Trümper[1], D. Kube[1], M. Vockerodt[6]. [1]*Georg-August University Göttingen, Hematology and Oncology, Göttingen, Germany,* [2]*Dana-Farber Cancer Institute, Department of Biostatistics and Computational Biology, Boston, USA,* [3]*University of Regensburg, Institute for Functional Genomics, Regensburg, Germany,* [4]*Charité Berlin, Institute for Pathology, Berlin, Germany,* [5]*Birmingham Children's Hospital, Department of Paediatric Otolaryngology, Birmingham, United Kingdom,* [6]*University of Birmingham, Cancer Research UK Division of Cancer Studies, Birmingham, United Kingdom*

Background: Aggressive Non-Hodgkin Lymphoma (aNHL) are a heterogenous group of malignancies derived from germinal centre B (GC B) cells. Burkitt's lymphoma (BL) is the most homogeneous aNHL entity, characterized by an aberrant expression of the proto-oncogene c-Myc. Recently BL was defined by a specific gene expression signature including c-Myc as one hallmark. Despite an abundant number of cell line investigations and murine models, there is a lack of experimental systems to investigate the role of c-Myc for the transformation of human GC B cells. Therefore we expressed c-Myc in primary tonsillar GC B-cells and monitored expression changes using microarray gene expression profiling. We performed analyses integrating expression profiles from clinical lymphoma samples pointing us to potential mechanisms of disease initiation and progression. Furthermore we asked whether these changes permit to further subgroup aNHL.
Materials and Methods: Purified human tonsillar $CD10^+$ GC B cells were transfected with a c-Myc expression plasmid (treatment) or empty vector (control). mRNAs from 8 independent treatment-control pairs (8 human tonsils) were subjected to gene expression profiling (Affymetrix® U133 plus2.0). Gene set enrichment analysis (GSEA) and bioinformatics integration of two large clinical lymphoma micorarray data sets were used to generate a c-Myc gene expression signature.
Results: Microarray profiled genes were ranked by concordance of their expression levels with those of c-Myc in both tonsillar B cells and tumours. Gene set enrichment analysis revealed a strong enrichment of c-Myc target genes and a depletion of CD40/NF-κB pathway targets. We defined the c-Myc signature comprising the top c-Myc responding genes as c-Myc index. This index stratifies aNHL patients based on the expression of the c-Myc signature genes. The signature is consistently expressed in BL, while its expression varies in DLBCL. In two independent clinical DLBCL microarray data sets the presence of a high c-Myc index is significantly associated with a shorter overall survival.
Conclusion: Our approach integrates two important aspects of cancer research; intervention in experimental model systems and observation on tumour samples. Mimicking aberrant c-Myc expression in GC B cells provided us with insights into downstream molecular pathways affected, confirmed BL as unique disease entity, and yielded a novel prognostic stratification of DLBCL.

418 Differences in radiation, cisplatin, and cetuximab sensitivity between subpopulations of head and neck cancer cells

C. Nilsson[1], L. La Fleur[1], K. Roberg[1]. [1]*University Hospital, Otorhinolaryngology, Linkoping, Sweden*

Background: Mortality in head and neck cancer is high due to the emergence of therapy resistant local and regional recurrences that may originate from resistant cancer stem cells (CSCs). Cells with CSC characteristics in head and neck squamous cell carcinomas (HNSCCs) have been identified, for example, by the expression of CD44. However, no definite marker of CSCs in HNSCC has been presented. The aim of the present study was to identify cells with CSC characteristics in HNSCC cell lines and evaluate differences in sensitivity to radiation, cisplatin and the EGFR antibody cetuximab between subpopulations of cancer cells.
Material and Methods: A HNSCC cell line established at the division of otorhinolaryngology at Linköping University Hospital was used in passages 8−20. Experiments were approved by Linköping ethical committee. Cells were cultured in Keratinocyte Media supplemented with 1% fetal calf serum. Subpopulations of cells were detected and sorted after direct immunofluorescence staining of surface CD44 (CD44 bright/dim cells) or epithelial growth factor receptor (EGFR+/− cells), or vital staining of aldehyde dehydrogenase (ALDH) activity (ALDH+/− cells). Sorted cells were exposed to radiation (4 Gy), cisplatin (2 μg/ml for 1 h) or cetuximab (30 nM for 5 days). Ten days after sorting, treatment response was analyzed by a crystal violet assay.
Results: CD44 bright, EGFR−, and ALDH+ cells had a lower sensitivity to radiation, cisplatin and cetuximab compared to CD44 dim, EGFR+, and ALDH− cells. Differences in the morphology were found between CD44 bright and dim cells and between EGFR− and + cells, with CD44 bright and EGFR− cells displaying a more spindle shaped morphology. In contrast, ALDH+ cells were found to be larger and more round-shaped compared to ALDH− cells. Co-staining of the markers showed that the majority of EGFR− cells had a high CD44 staining while ALDH positive cells were found to be CD44 negative.
Conclusion: We here identify, in a recently established HNSCC cell line, subpopulations of cells using markers suggested to characterize CSCs; however, co-staining could not identify a single population of cells positive for all of these CSC markers. This may suggest heterogeneity among the CSC population. More importantly, all CSC-like subpopulations displayed a lower sensitivity to radiation, cisplatin and cetuximab compared to their respective control population.

419 V600EB-RAF cooperates in Epithelial to Mesenquimal Transition regulating E-Cadherin and ILK-1 expression through the MEK/ERK-MAPK pathway

P. Baquero[1], I. Sánchez-Hernández[1], A. Chiloeches[1]. [1]*Universidad de Alcala, Biochemistry and Molecular Biology, Alcala Henares, Spain*

B-RAF mutations have a high prevalence in papillary thyroid cancer (PTCs) and anaplasic thyroid cancer (ATC), ranging from 35 to 65%. The most commonly B-RAF genetic alteration in human PTCs is a thymidine to an adenosine transversion at nucleotide 1799 that leads to a V600E substitution. This ^{V600E}B-RAF mutation is important not only to initiate tumourogenesis, but also required for the maintenance and progression of PTCs to advanced stages of tumour, extrathyroidal invasion and metastasis, as well as correlated with increased levels of Epithelial to Mesenchymal Transition(EMT)-associated genes.
On the other hand, Integrin Linked Kinase (ILK) has been also linked to cancer, because its expression is also elevated in tumours and correlates with tumour stage and grade. Thus, although ILK is expressed in normal thyroid cells, its levels are much higher in thyroid tumours. Its role could be exerted through the down-regulation of E-Cadherin expression, leading to the subsequent EMT.
The aim of this work is to study new molecular mechanisms mediated by ^{V600E}B-RAF in the development of PTC and elucidate the implication of this mutation in the regulation of certain proteins involved in the EMT. For this purpose we used three thyroid cancer cell lines, two of them harbouring the ^{V600E}B-RAF mutation and another one expressing wild type B-RAF.
We observed that ^{V600E}B-RAF abrogation by using short interfering RNA, reduces the expression of ILK, measured by quantitative real time PCR assays and western blot. This decrease correlates with lower phosphorylation levels of

its substrate GSK3β and either restores or increases the levels of expression of E-Cadherin. Moreover, the treatment with the MEK inhibitor U0126 induces similar effects to those observed after elimination of B-RAF expression, revealing that these effects are mediated by the canonical MEK/ERK-MAPK pathway. Furthermore, cell migration and invasion decrease when B-RAF expression and MEK activity are inhibited in these cells.
All these data show that ^{V600E}B-RAF plays a key role in EMT of thyroid cancer cells by increasing ILK expression and the concomitant decrease of E-Cadherin levels. These and further investigations may contribute to explain the role of ^{V600E}B-RAF in thyroid cancer progression and will help to understand the biological processes involved.

420 Impact of altered expression of E2Fs gene family on tumourigenic and phenotypic process in human colon cancer

M. Voegelin[1], E. Guérin[1], N. Hammelin[1], C. Nicolet[1], M.P. Gaub[1], D. Guenot[1]. [1]*Louis Pasteur University, EA4438, Strasbourg, France*

Background: In order to identify molecular genomic markers predictive of initiation and/or progression of human colon cancer (CC), a genome-wide analysis highlighted a micro-deletion at the 1p36.11−12 region in 23% (n = 115) and 47% (n = 59) of adenomas and carcinomas, respectively. Also, RT-QPCR evidenced a E2F2 transcript decrease in human colon tumours. Within the micro-deleted region, a potential target gene, E2F2 is described as either oncogenic or tumour suppressor, depending on the tissue or cell type. Thus, the aim of this study was to specify the functions of some of the E2F genes in CC, and the impact of the E2F2 deletion in the human colon carcinogenesis process.
Material and Methods: Two activators, E2F1 and E2F2, and one repressor, E2F4 were over-expressed in the human CC cell line TC7 (a well-differenciated subclone of Caco-2 cells) via stable transfection with plasmids containing the cDNA of each gene or the cDNA coding for the green fluorescent protein as a control. Survival (clonogenic assay) and migration properties (wound healing assay) of the cells were then assessed. In parallel, these cells were subjected to RNA interference experiments using siRNA directed against E2F1, -2 and -4. Transfection efficiency was validated by RT-QPCR and Western blot and phenotypic studies evaluated by immunocytochemistry.
Results: The number of colony-forming cells and the cell migration process were decreased in cells overexpressing E2F1 or E2F2 and to a lesser extent in those overexpressing E2F4.
E2F1, -2 and -4 silencing induced gene-specific morphological modifications, notably by modulating the expression of markers involved in differentiation, cytoskeleton organisation and cell-cell junctions. In addition, alteration of E2Fs expression in tumour colon cells induced compensation/regulation mechanisms between E2Fs themselves and with known associated proteins such as the pocket protein pRB and their dimerization partner DP. Finally, the tumourigenic potentiel of E2Fs overexpression has been evaluated by subcutaneous injections of the transfected cells into nude mice.
Conclusions: These data suggest that in colon TC7 cells, altered expression of E2F2, as well as of two other E2Fs members, brings the cells to change their migratory, survival and phenotypic properties asssociated with disseminating process. These results will be confronted with the on-going *in vivo* xenograft model experiments to strengthen the hypothesis that E2F2 expression deregulation could play a key role in human colon tumour initiation/progression.

421 The OPCML tumour suppressor functions as a repressor-adaptor, negatively regulating receptor tyrosine kinases in ovarian cancer

A. McKie[1], S. Vaughan[1], I. Okon[1], E. Zanini[1], F. Al-Jayoosi[1], R. Agarwal[1], E.W.F. Lam[1], H. Gabra[1]. [1]*Imperial College London, Oncology, London, United Kingdom*

Background: We previously identified opioid binding protein cell adhesion molecule (OPCML) as epigenetically inactivated in 83% of ovarian cancers demonstrating it was a functional tumour suppressor *in vitro* and *in vivo*[1]. OPCML belongs to the "IgLON" family of glycosylphosphatidylinositol (GPI)-anchored cell adhesion molecules with 3 extracellular I-type immunoglobulin domains[2–4]. The GPI anchor may sequester IgLONs to cholesterol-rich "rafts" that could confer specificity of signaling capability and choice of interacting partners[5]. We hypothesized that OPCML might interact with trans-membrane receptor tyrosine kinases (RTKs) and abrogate growth factor mediated signalling.
Materials: The ovarian cancer line SKOV3 shows very low expression of OPCML and is heavily hypermethylated. Stable transfected OPCML lines (BKS2.1 & SKOBS3.5) as well as vector-only control (SKOBS-V1.2) were used to identify differences in RTK expression and phospho-signaling upon growth factor stimulation. RNAi strategies in the normal ovarian surface epithelial line OSE-C2[6] were generated to look at the activation RTK signaling upon depletion of physiological OPCML. Interaction with RTKs in co-IP experiments used the pAb (R&D Systems) in SKOV3 cell lines. Refinement of the OPCML-RTK interactions was carried out using nGST-OPCML fusion proteins with either cell lysates or *in vitro* with expressed RTK fragments.
Results: We show that OPCML interacts with and downregulates HER2 and FGFR1, leading to inhibition of those signaling pathways in ovarian cancer cells. siRNA knockdown of OPCML in OSE-C2 strongly upregulated HER2 with concomitant increase in EGFR signaling. Interrogating multiple RTKs upon constitutive OPCML expression in SKOV3 cells or knockdown in OSE-C2 has highlighted FGFR1 as another target for interaction and down-regulation. The *in vitro* translated HER2 ECD interacted with GST-OPCML protein, but not a truncated fusion protein with the 3rd juxtamembrane Ig domain (Ig-III) deleted suggesting Ig-III is crucial for interaction with HER2. Exploring whether OPCML interacted with FGFR1 & 2 suggested both nGST-OPCML and the truncated version interacts with FGFR1, therefore Ig-III is not essential for FGFR1 binding to OPCML.
Conclusions: The OPCML tumour suppressor functions by negatively regulating HER2 and FGFR1, abrogating their pro-oncogenic functions. This has general implications for understanding the relationship of IgLONs to the RTK pathways, and their role in cancer biology.

Reference(s)

[1] Sellar, G. C., Watt, K. P., Rabiasz, G. J., Stronach, E. A., Li, L., Miller, E. P., Massie, C. E., Miller, J., Contreras-Moreira, B., Scott, D., et al. (2003). OPCML at 11q25 is epigenetically inactivated and has tumour-suppressor function in epithelial ovarian cancer. Nat Genet 34, 337–343.
[2] Schofield, P. R., McFarland, K. C., Hayflick, J. S., Wilcox, J. N., Cho, T. M., Roy, S., Lee, N. M., Loh, H. H., and Seeburg, P. H. (1989). Molecular characterization of a new immunoglobulin superfamily protein with potential roles in opioid binding and cell contact. EMBO J 8, 489–495.
[3] Hachisuka, A., Nakajima, O., Yamazaki, T., and Sawada, J. (2000). Developmental expression of opioid-binding cell adhesion molecule (OBCAM) in rat brain. Brain Res Dev Brain Res 122, 183–191.
[4] McNamee, C. J., Reed, J. E., Howard, M. R., Lodge, A. P., and Moss, D. J. (2002). Promotion of neuronal cell adhesion by members of the IgLON family occurs in the absence of either support or modification of neurite outgrowth. J Neurochem 80, 941–948.
[5] McKie, AB, Okon I, Vaughan, S, Shaposhnikov, D, Wong, J, El-Bahrawy, M, Lam, E, Stebbing, J and Gabra, H(2010). The OPCML Tumour Supressor Binds HER2 Abrogating EGF Mediated Signaling and Enhances Sensitivity to Clinical Inhibitors of the HER Pathway in Ovarian Cancer. Submitted to J. Clin. Invest.
[6] Davies, B, Steele, I, Edmondson, R, Zwolinski, S, Seretzki, G, Zglinicki, T, O'Hare, M. (2003). Immortilisation of human ovarian surface epithelium with Telomerase and temperature-sensitive SV40 large T antigen. Exp. Cell Res.

422 LPA receptor 4 mediates autotaxin-induced invadopodia production and invasion

K. Harper[1], D. Arsenault[1], S. Boulay-Jean[1], A. Lauzier[1], F. Lucien[1], C. Dubois[1]. [1]*Centre Hospitalier Universitaire de Sherbrooke, Paediatrics (Immunology), Sherbrooke, Canada*

The ability of cancer cells to invade and metastasize is the major cause of death in cancer patients. Recent studies indicate that tumour cell invasion and metastasis may be initiated by the formation of the actin-rich cell protrusions, invadopodia. Autotaxin (ATX) is a secreted lysophospholipase whose expression level within tumours correlates strongly with their aggressiveness and invasiveness. ATX produces LPA, a phospholipid with known tumour promoting functions that acts through the G-protein coupled receptors, LPA_{1-6}, although only LPA_{1-3} have been linked to tumour progression. The purpose of this study was to assess the implication of ATX and LPA signaling in tumour cell invasion and invadopodia production.
We used the invasive fibrosarcoma cell line, HT1080, in fluorescent matrix degradation assays to study the influence of ATX on invadopodia production. Our results using HT1080 cells stably transfected with ATX or shRNA targeting ATX indicate that ATX is implicated in the production of invadopodia resulting in an increase in both their formation and function. By adding LPC or LPA, the substrate and product of ATX, to our assays we demonstrated that invadopodia production is dependent on the production of LPA from LPC. Among the LPA receptors LPA_4 has the highest expression in HT1080 cells. Using LPA_4 shRNA as well as agonists and inhibitors of the cAMP pathway we provide evidence that LPA_4 signaling through the cAMP-EPAC-Rap1 axis regulates invadopodia formation downstream of ATX. Furthermore, inhibition of Rac1, a known effector of Rap1 and invadopodia formation, abolished EPAC-induced invadopodia production, suggesting downstream participation of Rac1. Finally LPA_4 knockdown was further correlated with a decrease in cell invasion and in vivo metastasis in a 3D assay and a lung metastasis assay, respectively.
Our results suggest that ATX through LPA_4 is a strong inducer of invadopodia formation that correlates with the ability of the cells to invade and metastasize. This study also revealed an unexpected signaling pathway for cell invasion involving LPA_4-driven cAMP production and subsequent activation of the EPAC-Rap1-Rac1 axis.