496 Inhibition of Akt pathway restores the sensitivity to cetuximab in a head and neck squamous cancer cell line

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The Epidermal Growth Factor Receptor (EGFR) is a central regulator of cell proliferation in human cancers and is frequently overexpressed in many types of tumours. Cetuximab is a monoclonal antibody designed to block the EGFR activation, to induce the internalization of the receptor and to reduce the signaling pathway. However many tumours exert resistance to EGFR inhibitors. Accumulating evidence indicates that the response of cancer cells to cetuximab is a complex process that can be affected by multiple resistance mechanisms.
The aim of this study was to investigate the mechanisms of resistance to cetuximab treatment *in vitro.* We investigated the EGFR pathway, the expression of HER3 and HER ligands, the EGFR internalization after cetuximab treatment, alone or combined, and we compared results in CAL33 (HNSCC) and A431 (epidermoïd cancer) cell lines which present different sensitivities to cetuximab.
Cetuximab induced a high growth inhibition and a high inhibition of ERK and AKT phosphorylation in A431. By contrast, cetuximab induced a lower growth inhibition, an ERK phosphorylation inhibition but any inhibition of the AKT pathway. The cetuximab sensitivity of these cell lines was thus different and the difference would be based on the AKT pathway. To verify this hypothesis, we used an EGFR tyrosine kinase inhibitor and several AKT inhibitors. Results have shown that the AKT pathway can be inhibited in CAL33 cell line by an EGFR tyrosine kinase inhibitor and an AKT inhibitor. While the cetuximab induced a strong growth inhibition in A431, this growth inhibition was weakly increased by the combination. In contrast, cetuximab induced a weak growth inhibition in CAL33 while the growth inhibition was much stronger with the combination than in A431. We investigated the EGFR internalization role in these models. Preliminary results showed significant differences between these two cell lines.
In conclusion, this study had shown some explanations to the limited efficacy to cetuximab in CAL33. Firstly, we showed a persistent activation of AKT in CAL33 which might prevent the antitumour effect of cetuximab. Secondly, we have shown that the EGFR internalization and signal transduction mediation might contribute to the response to cetuximab in CAL33 and A431. The AKT pathway appears as a central element in the cetuximab sensitivity in these models and the combination of cetuximab with an AKT inhibitor could be a good therapeutic option in HNSCC.

497 Liver microenvironment stimulates aggressiveness of colorectal tumour cells more efficiently than matched primary tumour microenvironment – Hepatic CAFs induce ERK-mediated modification of cell morphology

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Background: Carcinoma-associated fibroblasts (CAFs), the more abundant cells in tumour stroma, are important contributors in determining the tumour's fate by establishing paracrine communication and secreting factors that enhance tumour progression. This study aimed to assess the influence of liver microenvironment on the progression of colorectal carcinoma.
Material and Methods: Conditioned media (CM) from matched normal colonic fibroblasts (NCF), CAFs from primary tumour (CAFpt) or liver metastasis (CAFlm) was obtained from a patient with colorectal cancer. We performed proliferation, colony formation, migration and invasion assays in DLD-1 and SW480 cells. Microarray and GSEA-analysis were performed in DLD-1 cells cultured in different CM. Proteomic evaluation of soluble factors secreted by fibroblasts was also carried out.
Results: Whereas CM from CAFpt and NCF increased proliferation of DLD-1 and SW480 cells, liver CAFs (CAFlm) induced an inhibition in relation to control DMEMF12. CM from hepatic CAFs (CM CAFlm) encouraged a more aggressive phenotype in colorectal cells determined by stimulation of motility, migration and invasiveness and resulted in cells differentiating towards a "neuronal-like" morphology, showing an extended formation of invadopodia and lamellipodia. Such changes correlate with a sustained activation of the ERK-pathway in DLD-1 cells. Differential transcriptomic profile of DLD-1 cells treated with conditioned media from CAFlm depicted overexpressed genes like ARF6, ACTR2 and RHOB that correlate with such morphology and in addition, associated with a prognostic signature of colorectal carcinoma in a GSEA-analysis. Proteomic analysis showed TIMP-1, SPARC, PAI-1 and collagen-a-1 as the more relevant exclusively detected in CM CAFlm. Validation in an independent set of 16 NCF, 16 CAFpt (10 paired), 3 hepatic stellate cells and 6 CAFlm (2 paired) and in 40 matched normal colonic mucosa/primary tumour/liver metastasis specimens showed overexpression of PAI-1, TIMP-1 and collagen-a-1 but not SPARC in liver microenvironment.
Conclusions: We describe that under the same genetic background, liver microenvironment provides more favourable conditions for colorectal cells to become more aggressive.

498 Telomere length as indicator of transposon silencing and cell genome stability

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Background: We discovered recently that 5′-CG-3′ and 5′-CNG-3′ sites are found in interfering RNAs (RNAi) sequences more often than they should be found in random DNA sequence. Therefore we hypothesized that binding of complementary DNA sequence by RNAi leads to recruiting of DNA methyltransferases that methylate *de novo* cytosine in 5′-CG-3′ and 5′-CNG-3′ sites of DNA, which appeared to be bound with similar sites in the RNAi sequence. As microRNA genes are the transposable element derivates, we supposed that mechanism of RNAi-directed DNA methylation appeared in the evolution with the purpose of silencing of the mobile genomic elements. Specific set of RNAi expressing in the stem cells can restore initial profile of their epigenetic markers through this mechanism, thus minimal level of the transposons activity is achieved and immortal status of stem cells is supported for ever.
Material and Methods: Prediction of microRNA targets within transcripts of stage-specific genes using TargetScan software (http://www.targetscan.org/).
Results: Transcripts of great number of stage-specific genes are the targets of the cell microRNAs. Therefore cell differentiation, starting with the earliest stages, must be accompanied with repression of some microRNA genes, otherwise these microRNAs would prevent expression of the stage-specific genes.
Conclusions: Differentiating cells can lose slowly the repressive chromatin markers because of the silencing of microRNAs genes that are essential for renewal of these markers through the RNAi-directed DNA methylation. This will excite the derepression of silent transposons with time, subsequent increase of level of DNA damages induced by them and following activation of cell DNA repair system including mechanisms based on homologous recombination. In our opinion, these mechanisms cause not only the DNA repair, but also illegitimate recombinations in telomere caps, since they are pre-recombination structures. As a result, the T-loops converse into rings and, accordingly, telomeres are shortened for the length of the lost circled DNA (50–500 bp) that exceeds few times DNA loss over the end-replication problem (3−5 bp).
This process can cause exhaustion of telomeres in cells, in which the activation of recombination process becomes apparent. Usually, telomere shortening has to correlate with duration and intensity of repair system activity, i.e. with severity of DNA damage and, consequently, probability of cell transformation. Thus, proliferative ability of these compromised cells is restricted. The telomere length is integral indicator of genomic stability in normal cells.
Apparently, large quantity of organism cells reaches with age the threshold of illegitimate activation of silent mobile genomic elements. Following apoptosis of most of these cells causes the ageing as biological phenomenon, while the transposon-mediated transformation of their part determines correlation between ageing and cancer appearance.

499 Prox1 expression in liver metastases from colon carcinoma

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Background: Scattered data reported the Prox1 involvement in dysplastic transformation of colonic adenomas on murine experimentral model and its role in progression of colonic adenocarcinomas. No or rare Prox1 positive cells were found in normal colonic mucosa especially in basal cells. The aim of this work was to identify presence and characteristic features of Prox1 positive cells in liver metastasis from colon carcinomas in human specimens.
Material and Methods: Our study included 12 liver biopsies from patients previously diagnosed with colon carcinoma (7 metastases from well differentiated adenocarcinomas, 3 from moderate differentiated carcinoma and 2 from poorly differentiated colon carcinoma). Resected liver specimens obtained by open surgery were fixed in 10% bufferred formalin and parrafin embedded. Immunohistochemistry was performed by using Prox-1 polyclonal antibody followed by incubation with ADVANCE/HRP system and 3,3 diaminobenzidine as chromogen.

Results: Seven of 12 liver metastases from colon carcinoma (58.33%) were positive for Prox1. We noticed Prox1 positive cells with nuclear expression pattern in tumour cells of liver metastasis from well (4 positive cases), moderately (2 positive cases) and poorly differentiated adenocarcinoma (1 positive case). We found a heterogenous distribution and different intensity of Prox1 expression. The highest number of Prox1 positive cells was observed at the edge between restant liver tissue and metastases. Few Prox1 positive cells were present in intratumoural area also, but with low intensity expression. Rare Prox1 positive reaction was observed into the cells lining vascular structure from tumour area.
Conclusions: To our knowledge, this is the first study which reported the presence of Prox1 positive cells in tumour cells of human liver metastases from colon carcinoma. Their distribution at the edge between liver tissue and tumour area suggests their involvement in the progression of liver metastases. Further studies will be needed to demonstrate the mechanism of Prox1 involvement in this type of liver metastasis. Our evidences concerning Prox 1 expression in vascular structures from liver metastases areas were not strong enough to support a lymphangiogenic process but it could be launch the question if such type of metastases are able to produce their own lymphathic vessels.

500 Anti-tumour effects of zoledronic acid and somatostatin analogues in murine androgen-independent neuroendocrine carcinoma as hormone-refractory prostate cancer model

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Background: The treatment strategy for patients with hormone-refractory prostate cancer (HRPC) eventually emerging during androgen deprivation therapy presents many challenges to oncologists. Neuroendocrine (NE) cells in cancerous tissue have biological and histological features that might affect progression to hormone-refractory status. Previously, we developed an NE allograft (NE-10) and its cell line (NE-CS) from the prostate of the LPB-Tag 12T-10 transgenic mouse. We demonstrated that NE carcinoma promoted the pulmonary metastasis of human prostate cancer cell line LNCaP as well as androgen-independent growth of LNCaP. It was hypothesized that NE cells could be a therapeutic target for HRPC. To clarify the treatment options for HRPC, we investigated whether NE-10 and NE-CS were controlled by several agents, including zoledronic acid (ZOL) and somatostatin analogues such as octreotide (SMS) and pasireotide (SOM), having potential anti-tumour activity.
Material and Methods: Nine-week-old male BALB/c nude mice, which were castrated and inoculated subcutaneously (s.c.) with a 50 mg tissue block of an NE-10 tumour, were treated for 6 weeks with ZOL (3 μg/body/week s.c.), SMS (2 μg/body/day s.c.), SOM (4 μg/body/day s.c.), ZOL plus SMS, ZOL plus SOM, or saline (an equal volume of solvent/day s.c.). The effects of treatment on tumour growth were determined by measuring tumour volume. *In vivo and vitro*, tumour cell apoptosis and proliferation were determined by immunohistochemistry using TdT-mediated dUTP-biotin nick-end labeling (TUNEL) and a Ki-67 antibody, respectively.
Results: Growth of NE-10 tumours in mice treated with ZOL, ZOL plus SMS, or ZOL plus SOM was significantly slowed compared to the saline control ($p = 0.003$, $p < 0.001$, $p = 0.001$, respectively). The number of TUNEL-positive cells per 1000 NE cells was significantly increased in tumours from mice treated with ZOL, ZOL plus SMS, or ZOL plus SOM compared to the saline control (means: 9.2, 11.6, and 12.7, respectively, vs. 2.4, $p < 0.001$). In contrast, the number of Ki-67 positive cells per 1000 NE cells was significantly decreased in tumours from mice treated with ZOL, ZOL plus SMS, or ZOL plus SOM compared to the saline control (means: 5.3, 8.3, and 4.2, respectively, vs. 15.9, $p < 0.05$). *In vitro*, ZOL induced time- and dose-dependent growth inhibition and apoptosis of NE cells involving Ras/MAPkinase pathway via mevalonate pathway inhibition. Neither SMS nor SOM induced growth inhibition of 50% or greater in NE cells.
Conclusions: These results suggest that ZOL induces growth inhibition and apoptosis of murine androgen-independent NE carcinoma, which supports the possibility that ZOL can be an effective therapeutic agent for HRPC.

501 Analysis of the contractility of prostatic cancer-associated fibroblasts in a 3D collagen gel contraction assay

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Background: In recent years, the stroma of several cancer types has been recognized as a source of pro-tumourigenic signals mediated via e.g. paracrine signaling from cancer associated fibroblasts (CAFs) or physical stimuli such as tissue stiffness. Tissue stiffness can be regulated by e.g. lysyl-oxidase (LOX), as this enzyme cross-links proteins of the extracellular matrix, and has been shown to promote tumour progression.
Our aim was to investigate the effects of TGFβ1 protein and neutralizing antibody, and the LOX inhibitor bAPN upon the contractility of prostate CAFs and non-cancer prostate fibroblasts using a collagen contraction assay (CGC).
Methods: CAF and non-cancerous fibroblasts were isolated via tissue culture from patients undergoing transurethral resection of the prostate, and used at passages 4–7 for CGC assays and proliferation assays.
Cell proliferation and cytotoxicity of compounds were assayed in 96 well plates with the MTS assay provided by Promega's CellTiterOne Assay.
The CGC assay serves as surrogate assay for tumour stiffness and CAF activity. Cells were used in 500 μl collagen type I gels in a 24 well plate format. After gel solidification, 1ml medium was added per well, the gel mechanically released and photographed 24 and 48 hours later. The area of gels was measured with ImageJ.
Results:
1. CAF were more contractile than non-tumourigenic fibroblasts
2. Non-tumourigenic fibroblast gels were inhibited in a dose-dependent manner by bAPN but not by TGFβ1 protein or a TGFβ1-neutralizing antibody (nAB).
3. Contraction of CAF gels was not significantly inhibited or enhanced by bAPN, TGFβ1 or TGFβ1-nAB
4. Proliferation assays: no significant effects upon growth were observed following treatment with bAPN, TGFβ1 protein or a TGFβ1-nAB

Conclusions: There was a significant difference between CAFs and non-tumourigenic fibroblasts in regard to their contractility and in responsiveness towards the LOX inhibitor bAPN. This suggests that tissue stiffness is influenced not only by LOX but also by other factors. Furthermore, it appears that CAF themselves can be resistant to treatment. However, this does not exclude the possibility that other compounds might actually prevent tumour stiffness and/or pro-tumourigenic signaling pathways.

502 Mammospheres phenotype in expressing hormonal receptors and triple negative breast cancer cell lines

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Background: Clinical behavior of breast cancer is influenced by several parameters, particularly the expression of hormonal receptors (HR) and HER2 overexpression.
The application of certain growth factors (as bFGF and EGF) *in vitro* develops groups of spherical colonies in suspension with stem cell properties designated spheres. In breast cancer have been identified a subpopulation with tumourigenic capacity. The expressing of $CD44^+/CD24^{low}$ is being studies as possibly stem cell markers.
The objective of this study is to characterize the CD44 and CD24 expression in cells exposed to bFGF and EGF. We aim to analyze the differential expression profile in breast cancer cell lines expressing HR as well as triple negative.
Material and Methods: The adherent cell lines MCF7 (HR positive) and HCC1806 (triple negative) were propagated according with ATCC recommendations. Subsequently cells were rinsed 10 minutes in trypsin-EDTA 0.25%. Mammospheres protocol consisted on cell cultured in 10 mL DMEM-F12 supplemented with 10 ng/ml bFGF and 20 ng/ml EGF. The medium of each culture was renewed every 2 days during 15 days. Afterwards were analyzed in a FACS Canto II flow cytometer, with monoclonal antibodies anti-CD44 and anti-CD24. The results were interpreted in the form of mean fluorescence intensity (MFI) for the receptors studied.
Results: The breast cancer cell line HCC1806 exposed to growth factors developed a sparse population of suspense cells. Comparing with controls not exposed to mammosphere protocol, the mammospheres expressed CD44 in a higher degree than the controls. This difference was substantial considering a subpopulation of suspense cells representing 1%. In HCC1806, the expression of CD24 decreased after mammospheres protocol, despite this difference being scarce.
Focusing on MCF7, the treatment with growth factors developed dense suspense groups of spheres. In this group, the cells gained considerable CD44 expression in most of the suspense population (99%), comparing with the controls. The expression of CD24 diminished on treated cells, but not markedly.
Conclusions: The mammospheres protocol formation developed a minority of cells $CD44^+/CD24^{low}$ in triple negative cell lines. On the contrary, this phenotype was more frequent in breast cancer cells expressing HR in the same conditions. These differential phenotypes may represent a higher stem cell population in HR positive than in triple negative breast cancer cells.