468 Role of the p14ARF tumour suppressor in EGFR-mediated growth control of lung cancer cells

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Background: Tyrosine Kinase Inhibitors (TKIs) that specifically block activity of the Epidermal Growth Factor Receptor (EGFR) are currently used in the therapy of lung cancer. Although responses to TKIs strongly depend on the presence of EGFR mutations (L858R, Del19), complete clinical response is rarely achieved. Having observed that expression of the p14ARF tumour suppressor is altered in human lung tumours with mutated EGFR (Mounawar et al, 2007, Cancer Res.), we postulated that p14ARF expression could counteract the growth properties of mutated EGFR lung tumour cells and play a role in TKIs response.
Material and Methods: Lung tumour cell lines with wild type or mutated EGFR (L858R) were transfected with p14ARF and/or mutant EGFR (L858R) encoding vectors. Cell growth was analyzed by methylene blue staining or cell counting. Apoptosis was evaluated after active caspase 3 staining (FACS). Expression of genes of interest was studied by western blotting and/or by quantitative PCR. Co-immunoprecipitation experiments were used to detect interactions between proteins.
Results: We show that p14ARF overexpression inhibits the growth of cells with constitutive or transfected mutant EGFR (L858R) by inducing apoptosis. We also provide evidence that p14ARF uses the STAT3 pathway to exert its negative control on cell growth, and induces by this way the downregulation of the anti-apoptotic protein Bcl2, a known target of STAT3. Preliminary results indicate that the histone acetyl transferase Tip60 could be involved in p14ARF-mediated Bcl2 downregulation. Moreover, we show that apoptosis induced by TKI exposure (Gefitinib) correlates with the accumulation of the p14ARF protein as well as with the downregulation of Bcl2 expression in EGFR-mutated (L858R) cells.
Conclusions: These results indicate that p14ARF protects cells from the antiapoptotic signals induced by mutated EGFR (L858R). They also suggest that p14ARF could play a role in the response to TKI.

469 Vitamin D signalling and metabolic pathways expression in breast cancer progression

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Background: Breast cancer is a heterogeneous disease associated with different patient prognosis and responses to therapy. Vitamin D has been emerging as a potential treatment for cancer, as it has been demonstrated that it modulates proliferation, apoptosis, invasion and metastasis, among others. It acts mostly through the Vitamin D receptor (VDR) and the synthesis and degradation of this hormone are regulated by the enzymes CYP27B1 and CYP24A1, respectively. We aimed to study the expression of these three proteins by immunohistochemistry in a series of breast lesions.
Materials and Methods: We have used a cohort of 947 samples, comprising normal breast, benign mammary lesions, ductal carcinomas *in situ* and invasive carcinomas and we have assessed the expression of the VDR, CYP27B1 and CYP24A1 by immunohistochemistry.
Results: The results that we have obtained show that all proteins are expressed in the various breast tissues, although at different amounts. The VDR was frequently expressed in benign lesions (93.5%) and its levels of expression were diminished in invasive tumours (56.2%). Additionally, the VDR was strongly associated with the oestrogen receptor positivity in breast carcinomas. CYP27B1 expression is slightly lower in invasive carcinomas (44.6%) than in benign lesions (55.8%). In contrast, CYP24A1 expression was augmented in carcinomas (56.0% in *in situ* and 53.7% in invasive carcinomas) when compared with that in benign lesions (19.0%).
Conclusions: From this study, we conclude that there is a deregulation of the Vitamin D signalling and metabolic pathways in breast cancer, favouring tumour progression. Thus, during mammary malignant transformation, tumour cells lose their ability to synthesize the active form of Vitamin D and respond to VDR-mediated Vitamin D effects, while increasing their ability to degrade this hormone.

470 Proteomic analysis of microvesicles released by the prostate cancer cell line PC-3

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Background: Prostate cancer is one of the most frequent cancer types in men with 679,000 diagnoses and 220,000 deaths worldwide each year [1]. There is a need for new strategies for prostate cancer diagnosis and treatment, and a strategy that has recently received some attention is based on microvesicles. It has been known for three decades that epithelial cells of the prostate gland release vesicles, so-called prostasomes [2]. Interestingly, prostasomes contain molecules acting on immunosupression, invasion, angiogenesis and malignant conversion and have recently been implicated in prostate cancer [3]. Moreover, molecules in prostasomes might be used as cancer biomarkers.
Material and Methods: We have performed a proteomic analysis of microvesicles released by the metastatic prostate cancer cell line PC-3 looking for proteins that may have this function.
Results: 435 proteins were identified by liquid chromatography/tandem mass spectrometry (LC-MSMS), 226 of these proteins were identified with more than 1 peptide. Several of the proteins identified in our study belong to the list of proteins commonly found in microvesicles [4], but other proteins seem to be specific to the vesicular population released by PC-3 cells. After Gene Ontology annotations, GO-cellular component, 23% of the proteins were classified as extracellular. Intracellular proteins were annotated in a variety of cellular compartments: cytosol, endosomes, nucleus, mitochondria, Golgi apparatus and endoplasmic reticulum. 30% of the proteins were annotated in the category cytoskeleton. After GO-Biological Process, the main annotations found were: transport, metabolic processes, cell communication, cell organization and biogenesis and regulation of biological processes.
Conclusions: Further characterization of the proteins found in prostasomes released by PC-3 cells is required to determine whether any of these proteins can be used as prostate cancer biomarker or can help us to understand the role of prostasomes in prostate cancer.

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471 Neurobiological studies of tumour progression

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Tumour progression represents very complex process. Cancer cells actively interact with their environment e.g. surrounding stroma and especially with the immune cells. Moreover, there are accumulated evidences that progression of cancer and development of metastasis is under tight influence of the nervous system. The system may monitor and modulate cancer growth directly and/or indirectly, via the immune system. Direct interactions between the cancer cells and nervous system is proved by several facts: (i) some types of tumour tissues are actively innervated; (ii) neurotransmitters may modulate certain steps of tumourigenesis; (iii) interruption or stimulation of nervous pathways influences cancer progression; (iv) cancer induces specific changes in activity of the brain of tumour bearing animals, as well as, cancer patients.
In the study, we investigated transmission of information between BP6 fibrosarcoma cells and the brain during various circumstances in experimental animals. Firstly, we analyzed the effect of chemical sympathectomy on survival of BP6 tumour-bearing rats. Moreover, we followed the effect of BP6 cells progression on the activity of selected brain areas.
We found that chemical sympathectomy, induced by application of neurotoxin 6-OH dopamine, prolonged survival of the BP6 tumour bearing rats. Moreover, we detected increased activity in certain brainstem and hypothalamic areas, including nucleus of the solitary tract, parabrachial nucleus and paraventricular hypothalamic nucleus in the rats administered intraperitoneally the BP6 cells.
Our data indicate that autonomic nervous system modulates progression of tumour growth and that information related to tumour progression is transmitted to the brain. These findings support neurobiological view of cancer based on assumption that the brain monitors, as well as, modulates cancer progression and development of metastasis.
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472 Interaction of ephrinB2 with its receptors EphB4 and EphB6 − potential impact on tumour-associated inflammation in human melanoma

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Background: Tumour-associated inflammatory cells (TAIC) are a major component of the tumour microenvironment and can contribute to both tumour progression and metastasis for instance by direct cell-cell interaction via membrane-bound proteins. Tumour cells show varying expression of Eph receptors and their ephrin ligands, which both are receptor tyrosine kinases.

Eph/ephrins are hypothesized to be possible mediators of tumour-associated inflammation. The aim of our study was to analyze the distribution of ephrinB2 and its receptors EphB4 and EphB6 in inflammatory and melanoma cells and to clarify proinflammatory effects due to Eph/ephrin-mediated cell-cell contact.

Material and Methods: HL-60 promyelocytes and THP-1 monocytes, differentiated into granulocytes and macrophages, were used as a model for TAIC. Undifferentiated and differentiated cells were co-cultivated with Mel-Juso and A2058 melanoma cells. EphrinB2, EphB4 and EphB6 mRNA expression and protein synthesis was investigated using qRT-PCR and flow cytometry. Secretion of the proinflammatory cytokines IL-6 and TNF-α was analyzed using ELISA.

Results: No alteration in gene expression of ephrinB2, EphB4 and EphB6 could be observed during differentiation of HL-60 and THP-1 cells. In contrast, protein synthesis of ephrinB2, EphB4 and EphB6 was two- to threefold higher in HL-60 granulocytes compared to HL-60 promyelocytes and HL-60 macrophages. THP-1 macrophages showed a slightly increased protein synthesis of EphB4 and EphB6 compared to THP-1 monocytes whereas ephrinB2 protein content remained constant. Co-culture of both THP-1 monocytes and macrophages with Mel-Juso cells caused a substantial increment in secretion of proinflammatory cytokines. Co-culture of both HL-60 granulocytes and THP-1 monocytes with A2058 cells did not affect cytokine secretion. By contrast, co-culture of HL-60 macrophages with A2058 cells resulted in increased IL-6 secretion whereas co-culture of THP-1 macrophages with A2058 cells resulted in increased IL-6 secretion but decreased TNF-α release.

Conclusions: To our knowledge, mRNA expression and protein synthesis of ephrinB2, EphB4 and EphB6 was investigated for the first time in undifferentiated and differentiated HL-60 and THP-1 cells and, moreover, in Mel-Juso and A2058 melanoma cells. Co-culture of TAIC with melanoma cells resulted in proinflammatory effects. To differentiate the role of various Eph receptors and ephrin ligands in mediation of these effects after direct cell-cell contact of TAIC and melanoma cells selective inhibitors for Eph are applied in ongoing studies.

473 Role of the transcription factor forkhead box p3 in breast cancer and metastasis

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Background: The transcription factor forkhead box P3 (FOXP3) up- or downregulates a large number of genes and it has been recently reported to be expressed in tumour cells. In our recent study FOXP3 expression in breast cancer was associated with worse overall survival probability and the risk increased with increasing FOXP3 immunostaining intensity. FOXP3 was also a strong prognostic factor for distant metastases-free survival but not for local recurrence risk; moreover the hazard ratio of FOXP3 expression and of lymph node positivity were similar.

We investigated the involvement of FOXP3 in the metastatic process using *in vivo* and *in vitro* models.

Material and Methods: Since human breast cancer cell lines hardly develop metastases in experimental models the human lung large-cell carcinoma H460 cell line, expressing high levels of FOXP3 was used. Silencing FOXP3 gene with small interfering RNA was performed and the effects of FOXP3 down-regulation on both proliferation and migration were evaluated.

Two FOXP3-silenced clonal lines and their correspondent mock cell lines were injected subcutaneously in SCID mice and the number of spontaneous lung metastases was compared between the two experimental groups.

A gene expression analysis on both FOXP3-knocked down clones and mocks was performed using the Illumina microarray platform.

Results: FOXP3 silencing resulted in a 70% down-regulation of both FOXP3 mRNA and protein.

H460 cell migration was significantly ($p < 0.0001$) impaired by siRNA-mediated knockdown of FOXP3 expression, whereas cell proliferation was not affected.

A significantly reduced number of lung metastases was observed in mice injected with two different FOXP3 silenced clones, as compared to control mice (clone1: $p < 0.0001$; clone2: $p = 0.0123$).

Several pathways with a well-known role in metastatic process and that might be up- (chemokine ligands and interleukine pathways) or down- (p53 signaling and cell adhesion molecule pathways) modulated by FOXP3 were identified by gene expression analysis.

Conclusions: Our data suggest that FOXP3 expression in tumour cells might be related to the metastatic potential.

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474 Phosphatidylcholine-specific phospholipase c as a new molecular target to weaken the effects of her2 amplification in breast carcinoma

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Background: In the present study we investigated the capability of the phosphatidylcholine-specific phospholipase C (PC-PLC) enzyme to regulate the molecular mechanisms controlling HER2 overexpression on membrane of breast cancer cells by altering, through the enzyme inhibition, the rates of endocytosis and lysosomal degradation of the receptor.

Material and Methods: Membrane localization and direct interaction of PC-PLC with EGFR family members (HER2, EGFR and HER3) were investigated both on HER2-overexpressing and non-overexpressing breast cancer cell lines, using several experimental procedures, such as confocal laser scanning microscopy, flow cytometry, extraction of lipid rafts and immunoprecipitation experiments. The effects of PC-PLC inhibition on membrane HER2 expression, and on the overall contents of HER2, HER2-HER3 and HER2-EGFR heterodimers were monitored in the HER2-overexpressing SKBr3 cells, following either transient or continuous receptor engagement with anti-HER2 monoclonal antibodies, including Trastuzumab.

Results: PC-PLC enzyme was found to selectively accumulate on the plasma membrane of HER2-overexpressing breast cancer cells, where it co-localized and interacted with HER2 in raft domains. Inhibition of this enzyme resulted into altered rates of HER2 internalization and lysosomal degradation, and induced down-modulation of HER2 expression on the plasma membrane. Besides, PC-PLC inhibition led to a strong retardation of HER2 re-expression on membrane and to a substantial decrease in the overall cellular contents of HER2 as well as HER2-HER3 and HER2-EGFR heterodimers. We also found that the PC-PLC inhibitor had a deep impact on SKBr3 cell proliferation.

Conclusions: Altogether, these data indicate that PC-PLC could play an important role in regulating both the HER2 endocytic pathway and HER2 amplification effects and suggest that, by weakening the oncogenic HER2-mediated signal, PC-PLC inhibition may offer additional ways to enhance the effectiveness of current therapeutic strategies against breast carcinoma.

475 Inhibition of phosphatidylcholine-specific phospholipase c as a new strategy to induce differentiation of breast cancer cells

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Background: Purpose of this study was to investigate whether inhibition of phosphatidylcholine-specific phospholipase C (PC-PLC), an enzyme involved in the differentiation and proliferation of mammalian cells, could be used as a potential antitumour strategy against breast cancer cells by affecting their differentiation and epithelial-mesenchymal transition (EMT), critical to tumour progression and malignant transformation.

Material and Methods: The expression of PC-PLC in intracellular compartments was analyzed by confocal laser scanning microscopy (CLSM) and western blot analyses in different epithelial breast cancer cell lines, ranging from non tumoural (MCF-10A) to highly invasive and metastatic cells, such as MDA-MB-231 cell line. PC-PLC activity was measured by Amplex Red assays. Lipid droplets production and composition were evaluated by flow cytometry, CLSM analyses, ^{1}H NMR spectroscopy and Thin Layer Chromatography (TLC). The expression of typical EMT markers was detected by western blot and CLSM analyses.

Results: PC-PLC more massively accumulated in intracellular compartments of tumour cell lines (MCF-7, SKBr3 and MDA-MB-231) than in non tumoural cells (MCF-10A). The PC-PLC activity was much higher (to 3−6X) in all the analyzed tumour cell lines than in non tumoural cells, the highest activity being detected in the MDA-MB-231 cells. Inhibition of PC-PLC activity was associated with cell growth inhibition, in the absence of apoptosis, and in the production of lipid droplets, a typical marker of breast epithelial cells maturation. Increases in cholesteryl esters and triacylglycerol were, in particular, detected in the MDA-MB-231 cells following 48 h and 72 h exposure to the PC-PLC inhibitor. Tumour cell lines incubated with this inhibitor also showed significant changes in the expression of typical EMT markers, such as downregulation of vimentin (marker of mesenchymal cells), galactin-3 and milk fat globular epidermal growth factor-8, while E-cadherin (marker of epithelial cells) was up-regulated.

Conclusions: These results highlighted the role of the PC-PLC enzyme activity in the proliferation of breast cancer cells as well as in their epithelial-mesenchymal transition, suggesting that PC-PLC inhibition could represent a powerful strategy to control tumour progression and malignant transformation in this tumour cells.