Sunday 27 June 2010 09:45–17:30

Poster Session

Experimental/Molecular Therapeutics, Pharmacogenomics

198 Exploring a new therapy for neuroblastoma: silencing of doublecortin-like kinase using RNA-interference

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Neuroblastoma is one of the most common childhood cancers. Microtubule-destabilizing agents are used in the treatment of these tumours. However, resistance to chemotherapeutic agents and systemic toxicity make neuroblastoma a difficult drug target.
In our previous work, we found that doublecortin-like kinase (DCLK) gene transcripts are crucial microtubule-associated proteins for correct proliferation and differentiation of neuroprogenitor cells. Gene expression profiling revealed a high expression of these transcripts in neuroblastoma patients. Furthermore, these transcripts are endogenously expressed specifically in neuroblasts but are not found in other cell types. Suppression of DCLK by short interfering RNA (siRNA) disrupted the mitotic spindles in neuroblastoma cells and gene expression profiling revealed numerous differentially expressed genes indicating apoptosis. Apoptotic cell death of neuroblastoma cells by DCLK knockdown was further confirmed by several assays. Interestingly, mitochondria were the most affected cell components after DCLK-long knockdown. We also found in human neuroblastomas a significant correlation between DCLK expression and genes related with mitochondria activity. Furthermore, we showed a successful delivery of siRNA targeting DCLK to neuroblastoma cells by using specific peptide-siRNA conjugates.
In conclusion, silencing of the DCLK gene by siRNA interference is a novel potential therapeutic approach for neuroblastoma with the promise of combining high specificity with fewer side effects. Peptide-siRNA conjugates might be the tool needed for specific neuroblastoma delivery.

199 IGFBP-3 promoter methylation activates the PI3K/Akt intracellular signaling pathway in CDDP resistant cell lines

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The purpose of this study is to investigate the mechanisms regulated by the *IGFBP-3* promoter methylation that induce tumour cell proliferation and loss of sensitivity to CDDP in non-small cell lung cancer (NSCLC). We have recently reported that reduction of *IGFBP-3* expression by promoter methylation is involved in the CDDP acquired resistance process in 3 matched CDDP sensitive/resistant human cancer cell lines and in a human cohort of 36 NSCLC patients, probably because CDDP also induces DNA methylation *de novo.* The biological significance of *IGFBP-3* is of great importance in controlling cell growth, transformation and survival as IGF-I binds to *IGFBP3* with stronger affinity than to its own receptor (IGFIR), blocking their interaction and abolishing the mitogenic and antiapoptotic actions. IGF-I is also able to activate EGFR receptor. Those tyrosine kinases receptors (IGFIR and EGFR), signal through the PI3K/Akt pathway, that plays a crucial role in cell growth, proliferation, and survival and is commonly upregulated during tumourigenesis, including NSCLC; although the precise mechanism is not well defined.
The present study is based on a panel of three-paired CDDP resistant/sensitive NSCLC (H23 and H460) and ovarian (41M) cancer cell lines, with different *IGFBP-3* promoter methylation status. We have studied the relation between (1) *IGFBP-3* gene expression levels and promoter methylation status measured by RT-PCR, and by bisulfite sequencing and methylation specific PCR (2) the activation of EGFR and IGFRI biological pathways through the analysis of the PTEN, AKT, pAKT, pEGFR, EGFR, pIGFRI and IGFR protein levels by Western-Blot and (3) response to CDDP by crystal violet survival curves. Our results suggest that loss of *IGFBP-3* expression by promoter methylation in tumour cells treated with CDDP may activate the PI3K/AKT pathway through the derepression of the IGFIR signaling, inducing a resistant phenotype to CDDP and increasing cell growth. This study provides information regarding first, *de novo* promoter hypermethylation of *IGFBP-3* and the development of CDDP resistance, through the activation of the cell survival pathway PI3K/AKT, and second, the potential use of *IGFBP-3* and PI3K/AKT members as biomarkers and targets enabling the diagnosis and a personalized chemotherapy treatment of NSCLC.
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200 Response of cancer cell lines to chemotherapeutic drugs: DNA repair phenotyping as an early cell- and drug-specific exposure marker

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Background: Resistance of tumours to cytotoxic agents is a major concern of cancer therapy. There is strong evidence that modifications of DNA repair pathways participate to therapeutic failure. However underlying mechanisms are poorly understood. In this study we investigated the earliness of the DNA repair response using a set of colorectal cancer cell lines treated by sub-toxic and toxic concentrations of different chemotherapeutic drugs. DNA repair is a complex network of several intricate pathways; in order to better address the DNA repair response, we used a comprehensive functional approach on dedicated biochips that allowed investigating Nucleotide and Base Excision Repair as well as Intra-Strand Cross-Links Repair simultaneously.
Material and Methods: Three colon cancer cell lines were treated for 48h with chemotherapeutic drugs at IC20, IC20/10 and IC20/100 (MTT test). Nuclear extracts were prepared and the DNA repair phenotype was established using an *in vitro* multiplexed DNA synthesis/excision repair assay on miniaturized support.
Two different parameters were investigated regarding DNA repair: the global DNA repair response and the contribution of each of the measured DNA repair pathways (toward photoproducts, 8oxoguanine, alkylated basses, cisplatin adducts, abasic sites and glycols) to the global response.
Results: Each cell line exhibited a specific repair phenotype at basal state (without treatment). Cytotoxicity and DNA repair response were not necessarily correlated. For almost all the cell lines and the treatments tested, the treatment impacted the DNA repair phenotype before any detectable cytotoxic effect. The precocity, the extent and the nature (inhibition or stimulation) of the repair response (modification with respect to basal state) were highly cell type- and drug-dependent.
Conclusion: Genetic background of the cell lines is heterogeneous and mutations in some important genes might drive the repair response. Our aim is now to combine view on DNA repair response, genotypes and cytotoxicity in the purpose of identifying predictive biomarkers of chemotherapy response. In addition, considering the complexity of the DNA repair mechanism responses, the use of a comprehensive approach is the most efficient strategy to decipher the relationship between DNA repair and drug response.
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201 The roles of STAT transcription Factors in imatinib resistance and sensitivity in BCR/ABL positive chronic myeloid leukemia cells

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Background: STAT proteins have important roles in carrying the information coming from extracellular signals to inside of the nucleus. Chronic myeloid leukemia (CML) is a hematological cancer resulting from the reciprocal translocation between 9th and 22nd chromosomes bringing the *BCR* and *ABL* genes together to form BCR/ABL protein. Imatinib binds to the ATP binding domain of the fusion protein and prevents the subsequent phosphorylation of the target proteins. Despite the survival periods of CML patients are prolonged by means of imatinib, in most of the cases, leukemia gains resistance and eventually, chemotherapy remains ineffective.
Aims: In this study, we aimed to identify the roles of STAT proteins in imatinib resistance and sensitivity in K562 cells, and revealing the effects of STAT siRNA suppression on cellular growth and apoptotic induction.
Methods: Expressions of *STAT* genes were assessed by quantitative real-time PCR (Q-PCR). For silencing the gene in both sensitive and 3 μM imatinib-resistant K562 cells (K562/IMA-3), HiPerFect Transfection Reagent was used. Cell proliferation was detected by XTT cell proliferation assay, and apoptosis was evaluated by changes in caspase-3 enzyme activity.
Results: Q-PCR analysis revealed that the *STAT5a* has the most significantly changing expression level among the others in K562-IMA-3 cells as compared to sensitive K562 cells. *STAT5a* expression increased by 67%, where *STAT5b* showed 56%, and *STAT3* showed 4% increases, respectively, as compared to sensitive K562 cells. The results of this study has also demonstrated that that silencing of *STAT5a* sensitized both sensitive and K562/IMA3 cells to imatinib. Transfected K562/IMA3 cells became almost 4.5-times more sensitive than the non-transfected counterparts while transfected sensitive cells showed approximately 1.12-fold increased sensitivity. These results

indicate that K562/IMA-3 cells are more effectively sensitized by silencing the *STAT5a* compared to the sensitive K562 cells. Non-transfected K562/IMA-3 cells showed 1.85- and 3.46-fold increases in caspase-3 enzyme activation in the presence of 5- and 10 μM of imatinib, while siRNA-transfected counterparts have shown 11.21- and 20.17-fold increases.
Conclusion: Observing these significant responses to imatinib after transfection with single gene-specific siRNA might provide new opportunities for dealing with the frequent occurrence of resistance to chemotherapeutic agents in leukemia.

202 Identification and evaluation of novel breast cancer related biomarker proteins by antibody proteomics technology

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Background: The identification of biomarkers is a promising approach for the diagnosis and effective therapy of cancer. In particular, disease proteomics is a potentially useful method for identifying such biomarkers. However, very few biomarker proteins for drug development have been discovered using this approach. The main difficulty is to efficiently select potential biomarkers from the many candidate proteins identified by the proteomics approach. To circumvent this problem, we have developed "antibody proteomics technology" that can screen for biomarker proteins by isolating antibodies against each candidate in a rapid and comprehensive manner. Here, we applied "antibody proteomics technology" to breast cancer-related biomarker discovery and evaluated the utility of this novel technology.
Material and Methods: *2D-DIGE analysis:* Cell lysates from breast tumour cells (SKBR3) and established normal breast cells (184A1) were labeled with Cy3 or Cy5 and analyzed by 2D-DIGE according to the manufacture's protocol. Non-labeled samples were also loaded in a normal gel for MS analysis and a modified gel that can be solubilized by sodium periodide for antibody isolation. Proteins of interest were extracted from the gel and subjected to MS analysis and used as targets for the phage antibody library.
Isolation of monoclonal antibody: Protein samples extracted from the solubilized gel pieces were immobilized onto a nitrocellulose membrane. Using these proteins as targets, phages displaying scFv antibody, which have affinity to the targets, were selected from non-immune scFv phage libraries.
Tissue microarray (TMA) analysis: Expression profiles of candidate proteins were analyzed by using breast tumour TMAs stained with the isolated scFv-phages.
Results and Conclusions: By 2D-DIGE analysis, 18 candidate proteins over-expressed in SKBR3 cells were identified. Using an *in vitro* scFv-phage affinity selection procedure, monoclonal scFvs binding to each of the 18 candidate proteins were successfully isolated within a few weeks. TMA analysis then identified novel biomarker proteins over-expressed in breast tumours and correlated with lymph node metastasis. Our data demonstrates the utility of the antibody proteomics technology for discovering and validating tumour-related proteins in pharmaceutical proteomics. We are currently analyzing the functions of the identified proteins as potential diagnostic markers or therapeutic targets.

203 Targeting the transcriptional activity of sarcoma specific chimeric fusion proteins

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Background: Alveolar rhabdomyosarcoma (aRMS) is an aggressive childhood sarcoma characterized by expression of the fusion protein PAX3/FKHR which is thought to drive malignancy of this tumour. Downregulation of the fusion protein induces apoptosis in aRMS cells supporting the notion that their survival depends on continuous expression of PAX3/FKHR. Hence, it could be hypothesized that small molecular drugs might already exist for the treatment of aRMS, but strategies to prioritize them have been lacking. We are trying to identify small molecules capable to specifically modulate the transcriptional activity of chimaeric oncogenic transcription factors in sarcomas.
Material and Methods: We screened a small compound library (LOPAC 1280, Sigma) which covers 1280 different drug-like and well annotated compounds covering all major drug types. We used an endogenous cellular model, Rh4, which represents a PAX3/FKHR bearing aRMS with a transcription profile very similar to tumour biopsies. As a read-out system we simultaneously assessed cell viability together with a well established and highly sensitive luciferase reporter assay based on the AP2beta target gene promoter to monitor fusion protein activity. Cells were plated into 96 well plates and treated with the compunds at a final concentration of 5 μM during 24 hours. We set as cut-off a 65% reduction in luciferase activity.
Results: We have obtained a list of nearly 53 promising compounds affecting the activity of PAX3-FKHR. Among them there are different types of molecules with a broad role of functions such as kinase inhibitors, topoisomerase II inhibitors or compounds related to nitric oxide. We have chosen a final list of 11 compounds that we have tested using different concentrations (5, 1 and 0.5 μM) and time points (24, 48, 72 hours) and finally we have decided to focus our attention in two compounds: fenretinide (retinoic acid p-hydroxyanilide) and the kinase inhibitor IC261.
Interestingly, both fenretinide and IC261 reduced the mRNA expression level of PAX3-FKHR and some well known target genes such as AP2β, fibroblast growth factor receptor 2 (FGFR2) or fibroblast growth factor receptor 4 (FGFR4).
Then, we analyzed the effects of both compounds in additional rhabdomyosarcoma cell lines, both alveolar and embryonal, measuring again the cell viability and expression levels of PAX3-FKHR and its target genes. Both compounds were effective in all aRMS cell lines (Rh41, RMS13) but not in cells of embryonal origin (Ruch-2, RD) that lack PAX3-FKHR expression.
Conclusion: Unlike most other experimental strategies which reason that an increased understanding of the biology (target genes) would lead to identification of active compounds, our strategy is a reverse approach that has allowed us to identify compounds that are not tested yet for aRMS treatment such as fenretinide and IC261, and that then in turn are expected to help understanding the biology of sarcomas, identify pathways critical for aRMS progression and lead to the development of new therapeutic strategies.

204 Identification and pre-clinical validation of surrogate soluble biomarkers correlating with therapeutic response to met inhibition

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Serum proteins may function as early and dynamic surrogate biomarkers of molecular sensitivity to targeted inhibitors in selected cohorts of patients. In a search for a panel of potential soluble biomarkers of response to Met receptor inhibitors in sensitive tumours, we pursued an unbiased screening approach followed by experimental validation using diverse Met-addicted models, both *in vitro* and *in vivo*. Two independent and complementary technological platforms – (1) a large-scale cDNA microarray profiling and (2) a medium-scale protein array – have been applied to the Met-addicted cell line GTL16 treated with the selective inhibitor PHA-665752. The intersection of the two datasets enclosed two candidate molecules robustly down-modulated upon treatment, interleukin-8 (IL-8) and urokinase-type plasminogen activator receptor (uPAR). By employing available ELISA kits to test for the concentrations of IL-8 and uPAR in the supernatants of GTL16 cells upon PHA treatment, we observed a significant reduction in the levels of IL-8 and of uPAR. Moreover, we performed the same experiments in two cellular models of resistance to Met inhibitors: (1) A549 cells and (2) GTL16 cells infected with the constitutively active Ras^{G12V}. In both models, Met inhibition negligibly influenced secretion of the two molecules. We performed serial blood sampling at day 0 (pre-treatment), 3, and 10, to assay the concentration of IL-8 and uPAR in the plasma of nude mice injected subcutaneously with GTL16 cells. We could detect a clearcut reduction in IL-8 levels over basal values at 72 hs, that persisted until the 10^{th} day of treatment; conversely, established xenografts of GTL16 expressing the Ras^{G12V} 'resistance gene' did not feature a similar reduction in IL-8 levels. We further extended our fishing analysis to six other molecules significantly modulated either in the microarray dataset (stanniocalcin-1 and REG4) or in the protein array (IL-6, GROα, MIF, and MCP-1) and in order to generalize the model, we gauged expression and dosage of such molecules in a tissue-specific panel of Met-addicted cell lines (MKN45, HS746T, and SNU5). ELISA-based analysis of such molecules indicated that two of them (GROa and IL-6) displayed consistent rmodulation in culture supernatants of Met-addicted cells following Met inhibition. These soluble proteins may warrant further investigation as surrogate plasma biomarkers of response to anti-Met targeted therapies in drug-responsive tumours.

205 In vivo delivery of siRNA to tumours and their vasculature by novel dendritic nanocarriers

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Background: New targets for RNAi-based cancer therapy are constantly emerging. Nevertheless, *in vivo* delivery of siRNA remains a crucial issue for its therapeutic success. We propose to encapsulate the siRNA in a cationic carrier system, which can strongly improve its stability, cellular uptake and silencing efficacy. We developed novel polymerized dendrimer core shell structures to deliver siRNA *in vivo*. These water-soluble macromolecular carriers accumulate in the tumour environment due to the enhanced permeability and retention (EPR) effect and therefore, represent ideal delivery vehicles for antitumour biological agents.