run. Each peptide is monitored by 4 transitions corresponding to major β- or γ-ion fragmentations. The integrated chromatographic peak areas for the transitions were summed and compared to summed peak areas for beta-actin or human serum albumin, which was used as normalization standards.
Results: We applied this approach to screen lung cancer biomarker candidates in a test set of 20 tissue samples from patients with and without lung cancer. The MRM analyses detected 9 candidate proteins. These candidates were differentially expressed in unfractionated tissue lysates from cases and controls. In addition to these, we have found that prefractionation of protein extracts or peptides derived from protein tryptic digests allows detection of lower abundance candidates.
Conclusions: Current efforts are focused on methodological and analytical refinements to confirm the optimum number of peptides, the number of transitions to monitor, and the applicability of this approach to serum or plasma samples. Our results suggest that this proteomic method may have potential for accurately quantifying candidate lung cancer proteomic biomarkers in complex biological specimens.
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P22

Efficient separation of plasma membrane proteins allowing identification of increased numbers of cell surface markers associated with breast cancer metastasis by comparative quantitative proteomics

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Background: The molecular mechanisms involved in the metastatic process of breast cancer cells are complex and incompletely understood, but cancer cell surface proteins seem to play a pivotal role in several steps. Defining the cell surface proteome in the metastatic context is, furthermore, of importance for identification of therapeutic targets. Traditionally, studies of protein expression have been restricted to examination of a small set of proteins, but recent advances in the field of mass spectrometry have enabled simultaneous analysis of large numbers of proteins in complex mixtures.
In this study we have examined two isogenic breast cancer cell lines, equally tumourigenic in nude mice, but exhibiting diametrically opposite metastatic capabilities. We developed efficient methods for isolation of cell surface proteins and analyzed these by comparative, quantitative mass spectrometry (MS) thereby identifying cell surface markers with altered expression pattern on metastatic vs. non-metastatic breast cancer cells.
Methods: The proteome of the metastatic cell line was metabolically labeled with C13 arginine and lysine by SILAC (stable isotope labeling by amino acids in cell culture). Cells from both cell lines were mixed in a 1:1 ratio and a crude membrane protein fraction isolated. The membranes and the proteins embedded herein were separated by Percoll/sucrose density gradient and fractions enriched in cell surface proteins and with little mitochondrial contamination are identified by enzymatic assays.
The cell surface proteins were enzymaticly digested and analyzed by LC-MS/MS. The proteins were identified and quantified by the VEMS 3.0 software.
Results: By using dual isotopic labels as compared to a single label the number of identified proteins that could be quantified were increased from less than 50% to more than 90%. As cell surface proteins generally are low abundant the percentage of identified proteins that were membrane proteins could be increased to 60% by analyzing each sample four times by LC-MS/MS. Using this method we have identified more than 1000 different proteins. Thirteen cell surface proteins have been identified as potential markers of metastatic breast cancer.
Conclusions: Inclusion of a Percoll/sucrose gradient provides an efficient mean of isolation of cell surface proteins with little contamination from other cellular compartments. Combined with the metastatic cell model, SILAC, and LC-MS/MS this protocol identifies potential targets for future drug development.

P4

Genes for normalization of qRT-PCR data in breast cancer

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Background: Quantitative real-time RT-PCR (qRT-PCR) has become a valuable molecular technique in basic and translational biomedical research, including cancer, and is about to become useful for clinical testing. To relate the obtained values between samples, the data needs to be normalized. This can be done in various ways; the most accepted being to internal, stably expressed, reference genes. Recently the traditionally used reference gene GAPDH has been shown to be influenced by the hormone oestradiol, while B2M may be influenced by factors present in brain tissue of alcoholics, emphasizing the need to identify the optimal genes to be used for normalization, within the tissue to be analyzed.
Methods: In this study we identified genes to be used for normalization of qRT-PCR data for estrogen receptor positive (ER+) invasive breast cancer (IBC) and also examined their applicability for ER− IBC, normal breast tissue and breast cancer cell lines. The reference genes investigated were RPLP0, TBP, PUM1, ACTB, GUS-B, ABL1, GAPDH and B2M, as well as the cytokeratin genes KRT14, KRT18 and KRT19.
Biopsies of 11 surgically removed ER+ IBCs, 4 ER− IBCs, 3 normal breast tissues and 3 ER+ cell lines were examined and the data analyzed by descriptive statistics, geNorm and NormFinder. In addition, the expression of selected reference genes in laser capture microdissected ER+ IBC cells, were compared with that of whole-tissue.
Results: TBP, RPLP0, PUM1 and ACTB were identified as the most suited for normalization of qRT-PCR data of ER+ IBC samples, as both geNorm and NormFinder consented on these. Further, TBP, RPLP0 and PUM1 were also identified by both programs for the collected group of human samples (ER+ and ER− BC and normal breast tissue).
Conclusions: In conclusion, these genes should be the reference genes of choice when performing qRT-PCR on normal and malignant breast specimens.

P30

Biological role of NHERF1 protein in breast cancer

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Background: NHERF1 is a PDZ domain containing protein that recruits membrane receptors/transporters and cytoplasmic signalling proteins into functional complexes. NHERF1 expression is altered in breast cancer but its effective role in mammary carcinogenesis remains undefined. We reported (Cardone RA, 2007) that NHERF1 overexpression in breast cancer is associated with invasion and aggressiveness of the disease. To further understand NHERF1 function and its biologic role in breast cancer, we analyzed NHERF1 protein expression in breast cancer patients.
Methods: Immunohistochemistry for NHERF1 was performed using EBP50 rabbit polyclonal antibody in 61 breast cancer patients. In particular, we examined 22 primary tumours from node negative (N0) patients, 19 primary tumours and metastatic lymph node from patients without distant metastasis (N1M0); 10 primary tumours together with metastatic lymph node and metastases from patients with distant metastasis (N1M1) and 10 carcinoma in situ (CIS). Moreover, NHERF1 protein expression was also evaluated in all normal tissue surrounding breast cancer. Colocalization of NHERF1 and HER-2neu was also investigated on high HER-2neu expression tumour tissues. Immunohistofluorescence for NHERF1 (polyclonal) and HER-2neu (monoclonal) was performed using the Alexa 488 goat anti-mouse IgG1 and 568 goat anti-rabbit IgG.
Results: NHERF1 positivity was present as membranous staining, especially at the luminal aspects of cells in normal epithelia, and as diffuse cytoplasmic staining in tumour and metastatic tissues. Interestingly, protein localization is strictly limited to the apical membrane region of the normal lobules, also when they are present in tumoral tissues.
A significantly higher NHERF1 cytoplasmic-expression and a lower protein membrane-expression have been found in tumour tissue with respect to normal ($p < 0.001$). Furthermore, NHERF1 cytoplasmic expression was higher in lymph node tissues with respect to normal ($p < 0.001$), while no difference was observed between tumour and metastatic tissues. These results have been confirmed in the different subgroups of patients.
Conclusions: Our study on human breast cancer tissues suggests that breast carcinogenesis is characterized by a different subcellular localization of NHERF1 protein from membrane to cytoplasm perhaps due to different binding with cell membrane. Ongoing immunohistofluorescence and confocal studies will further analyze the colocalization of NHERF1 and other target proteins.

P3

Randomised phase III clinical trial to evaluate the efficacy and safety of an integrated treatment (diet, pharmaco-nutrional and pharmacological) in cancer patients with cancer-related anorexia/cachexia and oxidative stress: interim results

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Background: In April 2005 a phase III randomised study was started to establish which was the most effective and safest treatment of cancer-associated anorexia/cachexia syndrome (CACS/OS) able to improve identified primary endpoints: increase of lean body mass (LBM), decrease

of resting energy expenditure (REE), increase of total daily physical activity, decrease of IL-6 and TNF-alpha and improvement of fatigue.
Methods: All patients were given as basic treatment: polyphenols + antioxidant agents alpha lipoic acid, carbocysteine, Vitamins E, A and C, all orally. Patients were then randomised to one of the following 5 arms: (1) Medroxyprogesterone Acetate (MPA)/Megestrole Acetate (MA); (2) Pharmaco-nutritional support containing 2 g EPA; (3) L-carnitine; (4) Thalidomide; (5) MPA/MA + Pharmaco-nutritional support + L-carnitine + Thalidomide. Treatment duration 4 months. The sample size was 475 patients. At May 2007, 160 patients, well balanced for all clinical characteristics, have been included. Body composition has been assessed by dual energy X-ray absorptiometry (DEXA) since January 2007.
Results: No severe side effects were observed. As for efficacy, an interim analysis on 145 patients showed an improvement of at least 1 primary endpoint in arm 3 (significant decrease of REE and fatigue), 4 (significant decrease of IL-6) and 5 (significant decrease of REE and fatigue), whilst arm 2 showed a significant worsening of LBM, REE and MFSI-SF. The t-test for changes demonstrated the worsening of LBM, REE and MFSI-SF in arm 2 versus arms 3, 4 and 5 and therefore it was withdrawn from the study. Arms 1, 3, 4 and 5 showed no statistical significant difference.
Conclusions: The interim results obtained so far seem to suggest that the most effective treatment for CACS/OS should be a combination regimen and L-carnitine alone. The study is still in progress up to completion of final accrual of 400 patients (sample size reduced because of withdrawal of arm 2 from the study).
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P54

Study of ATP7B copper transporter mRNA levels as a prognostic factor in advanced colorectal cancer patients treated with 5-fluorouracil plus oxaliplatin

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Background: It has been demonstrated a correlation between ATP7B protein and/or mRNA levels and response to treatment in ovarian cancer patients treated with cisplatin based chemotherapy. The aim of our work was to determine ATP7B mRNA expression levels in colorectal cancer (CRC) tumor tissue of patients treated with oxaliplatin (OXA) and 5-fluorouracil (5FU) according Spanish TTD group schedule and to correlate with response to treatment.
Methods: mRNA levels were analyzed by using Real Time PCR (RT-QPCR). The housekeeping gene used was â-actin and as a reference sample we used commercial human mRNA from liver. Chi-square and Fisher test were used in order to value differences in response rate to treatment. Time to progression (TTP) was studied by using Kaplan Meyer curves and Log rank test.
Results: Fifty-three advanced CRC patients treated with 5FU plus OXA were analyzed. 62.15% of them were males; primary tumour was localized in colon in a 66.7% of cases. We did not observe any progression to treatment in the group of patients that had ATP7B expression levels under percentile 25 (group 1). In contrast, patients with expression levels upper (group 2) showed 13.2% of progressions. Group 1 patients also had a greater number of complete responses than group 2 (33.3% vs 15.8%). TTP median was 14.66 and 7.18 months for group 1 and 2 patients respectively (Log rank p = 0.035).
Conclusions: According our results, lower expression of ATP7B in tumor tissue of CRC patients treated with 5FU plus OXA correlates with a greater response rate and a better TTP. However, these results should be confirmed in a higher number of patients.

P12

SELDI-TOF MS serum protein profiling predicts poor prognosis renal cancer patients

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Background: Approximately 30% of patients with renal cancer (RCC) present with metastasized disease and only 15−25% of patients respond to anti-tumor treatment. Although treatment outcome is improving due to the development of various targeted therapies, adequate selection of patients for these treatment approaches is important. Profiling of proteins in body fluids to predict patient outcome would be an attractive and non-invasive approach for utilization in the clinic. Surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) was used to identify protein signatures in the serum proteome of RCC patients that discriminate between patients with poor or good outcome.
Methods: In this pilot study we analyzed protein profiles in the serum of 57 renal cancer patients (2% stage I, 12% stage II, 7% stage III and 79% stage IV patients according to the American Joint Committee on Cancer) and 59 healthy controls. Denatured serum samples were incubated on CM10 ProteinChip arrays and analyzed using the PBS-IIC ProteinChip Reader. Clinical data was collected and the extended Memorial Sloan-Kettering Prognostic Factors Model for survival was calculated. Ratios discriminating between RCC cases and controls were selected to generate a predictive multi-protein model. Univariate and multivariate Cox Proportional Hazard analyses were performed. Protein masses included in the predictive model were identified.
Results: In RCC serum samples we identified ion masses predictive for patient survival, and built a protein-model consisting of five signature peaks with m/z ratios of 2944, 3331, 6457, 6654, and 9201 Da, that could correctly identify poor prognosis patients with sensitivity and specificity of 80% and 76% for 1-year survival. Cumulative 1-year survival was 93% for low-risk patients, compared to 48% for high-risk patients (P = 0.0001, Log-rank test). Multivariate analysis indicated that our model was an independent predictor of survival when compared to the Memorial Sloan-Kettering Prognostic Factors Model. The tentative protein identities were apolipoprotein C-I (doubly and singly charged, and a singly charged fragment), a haptoglobin-alfa1 fragment and a yet unidentified 2.9 kDa protein fragment.
Conclusions: SELDI-TOF MS can be used to assess the prognosis of RCC patients independent of present prognostic factor models.

P23

Novel computational paradigms in breast cancer familiarity profiling

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Background: Genomic DNA copy number aberrations are frequent in solid tumours although their underlying causes of chromosomal instability in tumours remain obscure. The aim of our work was to individualize a genomic profile characterizing familial breast cancer.
Methods: For this purpose, a series of 124 consecutive breast cancer patients analyzed for aCGH entered the study. The results have been elaborated by an Artificial Immune System paradigm that we hypothesized could be successfully employed in the elucidation of biological dynamics of cancerous processes using a novel fuzzy rule induction system for data mining (IFRAIS) of aCGH data.
The most important characteristic of IFRAIS is that it discovers fuzzy classification rules, naturally comprehensible. It is obvious, of course, that comprehensible knowledge is essential in real-world data mining problems (e.g. in bioinformatics). The accuracy of results are expressed in terms of medians of the extracted values. The selected strategy for training and validation was the KFold cross-validation with K = 5. The global level of accuracy reached by the system nears the 97%; a quite competitive result indeed, even if we consider that algorithms like J48 (C4.5 evolution) is not able to go beyond the 94.34% of accuracy.
Results: We evidenced 3 rules to define familial and 5 rules to define sporadic breast cancer. In particular, rule 1 for familial cases involved genes linked to neurodegenerative pathway and comprised genes involved in important step of cell cycle, including apoptosis and signalling transduction in processes activating NFKB (locus 20p13, locus 11q22.3), or with growth factor activity (locus Xq26.2). Rule 2 of sporadic cases included genes involved in cell metabolism and differentiation (TGIF, CYP2R1, PUM1), while rule 4 involved genes of apoptosis pathway and a gene encoding a protein highly conserved among species, SCOC, probably involved in regulation of important cell cycle processes.
Conclusions: In this work we present the study of a novel rule induction system. We can conclude that novel biologically-inspired data mining techniques seem to be competitive interesting tools in cancer research. However, the full understating of the underlying dynamics in cancer settlement and progression still remains a primary objective.

P82

18F-fluorothymidine(FLT)-PET as a biomarker of the antineoplastic effects of radiation therapy combined to the anti-angiogenic agent Enzastaurin in lung cancer model

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Background: Positron emission tomography (PET) with 15F-fluorodeoxy-glucose is an established tool in diagnosis, staging, and surveillance of cancer. A limitation of this imaging modality, however, is its nonspecificity