308 Poster

Methylation status of WIF-1 promoter region in breast cancer cell lines and tumour tissues

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Background: Aberrant epigenetic activation and upregulation of the Wnt pathway is a key feature of many cancers. Wnt inhibitory factor-1 (WIF-1) is a secreted antagonist that can bind to Wnt proteins directly and inhibit Wnt signaling pathway. Downregulation of WIF-1 protein expression has been detected in human bladder, breast, non small-cell lung, prostate and gastrointestinal carcinomas. WIF-1 has been reported to be silenced by promoter hypermethylation in human cancer cell lines and tissues.

Objectives of this study: To determine the presence of WIF-1 promoter methylation in breast cancer cell lines, tumour and non-tumorous tissues of breast cancer.

Methodology: Four breast cancer cell lines, MCF-7, T47D, ZR75–1 and MB231, were used. For the breast tissues, 20 surgically resected breast cancer tissues and the corresponding paired non-tumorous tissues taken more than 5 cm from the tumour at a macroscopically normal focus were analyzed (in case where such distance was not present, the non-tumour sample was taken from a distance furthest away from the tumour sample). Both the tumour and non-tumorous tissues were subsequently confirmed histologically. The tissues were snap frozen in liquid nitrogen-cooled isopentane and stored at −70°C until use. High molecular weight DNA was extracted from the breast tissues according to the method previously described. Methylation of the promoter region of WIF-1 was examined by bisulphite DNA modification followed by methylated specific PCR (MSP).

Results: CpG island hypermethylation in the WIF-1 promoter region was observed in 2 (50%) of 4 breast cancer cell lines, MCF-7 and ZR75–1. Analysis of the breast tissues revealed that 7 (35%) of the breast tumour tissues and 10 (50%) of the paired non-tumorous tissues had methylation of the WIF-1 promoter region.

Conclusion: WIF-1 promoter hypermethylation occurred at a high frequency in breast cancer tumour and non-tumorous tissues. This suggests the notion that WIF-1 methylation is an early and premalignant alteration, and supports previous reports that WIF-1 silencing due to promoter hypermethylation is an important mechanism underlying aberrant activation of the Wnt signaling pathway in carcinogenesis.

309 Poster

Expression microarray analysis of newly-developed chemotherapy-resistant breast cancer cell lines

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Chemotherapy resistance in metastatic breast cancer is a major challenge facing the treatment of this disease. Tumours can harbour a plethora of mechanisms to avoid the effects of chemotherapy agents such as increased membrane pump activity and drug detoxification enzymes. In order to investigate these and other possible mechanisms of chemotherapy resistance, we established novel breast cancer cell lines which were significantly resistant to the chemotherapy drugs doxorubicin, cisplatin and 5-fluorouracil. In contrast to other chemotherapy-resistant cell line models, these models of chemotherapy resistance aimed for a greater degree of clinical relevance by establishing resistant cell lines with concentrations of chemotherapy drug derived from patient's peak plasma concentrations. Secondly, treatment was carried out in discrete cycles, again to mimic the clinical environment. Control cell populations were grown in parallel to ensure that any genetic changes were due to chemotherapy resistance and not random changes, which can often occur in tumour cell lines.

For analysis of gene expression changes associated with in vitro chemotherapy resistance, RNA was extracted from resistant and control cell lines, reverse transcribed into fluorescently labelled cDNA and competitively hybridised to cancer-specific oligonucleotide microarrays. After normalisation and correction for dye bias effects, the expression a total of 17 genes (doxorubicin: 5; cisplatin: 7; 5-fluorouracil: 5) were found to be significantly altered between the chemotherapy-resistant cell lines and the control cell lines.

The development of these chemotherapy-resistant breast cancer cell lines and identification of candidate genes associated with in vitro drug-resistance may reveal novel therapeutic targets in breast cancer.

310 Poster

Detection of circulating tumor cells in peripheral blood of patients with primary and metastatic breast cancer

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Introduction: The purpose of this study was detection and analysis of disseminated tumor cells in blood of breast cancer patients by expression profiling using disseminated tumor cell (DTC) detection assay.

Material and Methods: Patients with primary breast cancer (n = 178) and patients with metastatic breast cancer (n = 161) were enrolled onto a multicenter clinical performance evaluation study. Five ml blood were used for DTC analysis. The DTC assay consists of immunomagnetic tumor cell selection (targets EpCAM and MUC-1). The immunobead selected cells were used for mRNA isolation and c-DNA synthesis. Breast carcinoma-associated transcripts GA733–2 (EpCAM), MUC-1 and HER-2 were analysed by multiplex PCR. Claudin7 was determined by single-round RT-PCR, while cytokeratin 19 (CK19), mammaglobin 1 (MBGB1) protate-specific ets factor (PSE) and survivin (BIRK5) were determined by nested RT-PCR. PCR products were analysed by capillary electrophoresis with the Agilent Bioanalyzer 2100. Specificity of the RT-PCR was confirmed by examination of blood of healthy donors.

Results: Sensitivity for every single transcript was adjusted to 2 tumor cells per 5 ml blood. In the group of patients with primary breast cancer (n = 174) we archieved an overall detection rate of 20%. In patients with metastatic disease 93 of 161 (58%) were positive. The detection rate of tumor-associated transcripts in primary breast cancer was 18% (18/101) for lymph node negative and 24% (18/76) for lymph node positive patients. Tumor-associated transcripts were heterogenously expressed in positive samples. Expression rates for MUC1, GA733–2, and HER-2 were 71%, 26%, 32%, respectively. Survivin, CK19, Claudin 7, MGB1, and PSE transcripts were detected at a frequency of 29%, 19%, 35% and 10%.

Conclusion: Using a new immunomagnetic tumor cell enrichment method we have established a c-DNA bank of peripheral blood samples from patients with primary breast cancer. Recently microarray strategies confirmed established and identified new informative molecular markers for the detection of micrometastatic cancer cells. In the present study we describe the application of a panel of 8 genes overexpressed at high levels in metastatic breast cancer for the identification of tumor cells in peripheral blood. HER-2, Survivin as a unique member of the inhibitor of apotosis protein family, as well as PSE identified in circulating breast cancer cells may serve as a prognostic indicators of tumor progression and could represent valid targets for new individualized therapeutic interventions.

311 Poster

Analysis of tamoxifen-resistance related genes by Taqman low density array technology in clinical samples

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Objective: Identifying distinct gene expression signatures of predictive diagnostic value is one of the challenges of genomics research. The study was performed to evaluate if a set of genes found to be differentially expressed in a tamoxifen-resistant and -sensitive human breast cancer xenograft model by means of Affymetrix GeneChip® analysis (Mol Cancer Ther 2005; 4(1):151–68) can be confirmed in clinical samples from breast cancer patients responsive to tamoxifen or developing a recidive under tamoxifen therapy. For this purpose we employed a new and efficient PCR technique combining Low Density Array with TaqMan® RealTime RT-PCR from Applied Biosciences.

Samples and Method: Samples from two independent animal experiments with the tamoxifen-sensitive human mammary carcinoma xenograft MaCa 3366 and its tamoxifen resistant subline MaCa 3366/TAM were used. Furthermore, we included 23 clinical samples including primary breast tumours of patients shown to be recurrent or to remain disease-free under tamoxifen treatment. We applied TaqMan® Low Density Array methodology to analyse the differential expression of 11 genes significantly up- and down-regulated in the tamoxifen-resistant tumour xenograft as compared to the sensitive one as determined by Affymetrix chips.

Results: Using TaqMan® Low Density Arrays we could show a high correlation of the xenograft data derived from Affymetrix chips and from Low Density Arrays. Several genes in the clinical samples of the recurrent

and the disease-free patient group showed the same trend of differential expression as they did in the xenograft model. These trends could be confirmed for all of the 5 down-regulated as well as for 3 of the 6 up-regulated genes. Comparing the expression data for each gene in individual patients, distinct expression differences were observed for NNAT, IGFBP5 and HOXB13.

Conclusion: TaqMan® Low Density Arrays provide an efficient and reliable RealTime PCR method in a 2 µl reaction volume for the simultaneous analysis of multiple genes. Expression data for several genes in clinical samples reveal differences with respect to recurrence under tamoxifen therapy. We were able to define candidate genes possibly related to tamoxifen responsiveness that will be subjected to further functional evaluation.

312 Poster

Gene profiling analysis of tissue-specific metastases from human breast cancer

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Breast cancers are prone to metastasise, particularly to the lymphatics, bone, liver, lungs and central nervous system. Once solid secondary tumours are established, the chances of long-term survival fall from over 90% to around 5%. The European project "MetaBre" investigates the molecular mechanisms of breast cancer organ-specific metastasis. The first step of this project has studied the gene expression profiles of tissue-specific metastases by use of microarray analysis (Affymetrix HG U133 plus 2.0, over 47,000 transcripts). To this aim, we hybridised 21 human breast cancer metastases resected from 5 different organs: lung (5), liver (6), bone (4), brain (4) and skin (2). We also analysed normal tissues from each corresponding target organ.

Statistical analyses were performed using BRB ArrayTools 3.2.2. "One class versus all" class prediction analysis was used to identify genes involved in organ-specific metastasis. 4 signatures of approximately 20 genes were established for lung, liver, bone and brain metastases. The validation of the signatures was performed by quantitative RT-PCR. These 4 signatures were able specifically to distinguish metastases from different sites from each other. Furthermore, our lung metastasis signature was able to discriminate breast cancer primary tumours relapsing to lungs from a series of tumours metastasizing to different organs. Thus, our expression profiling study allowed us to identify genes potentially involved in organ-specific metastasis.

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313 Poster

Gene copy numbers and expression of ErbB-1 and ErbB-2 in breast cancer

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Background: The family of ErbB oncogenes and their receptors play important role in breast cancer development, however prognostic relevance of ErbB-1 and ErbB-2 abnormalities is debatable. The aim of this study was to determine clinical impact of ErbB-1 and ErbB-2 gene copy numbers and expression in a large series of breast cancer patients.

Material and Methods: Study group included 225 consecutive stage I-III breast cancer patients treated between 1998 and 2002 in three Polish institutions. Average gene copy numbers (AGCN) of ErbB-1 and ErbB-2 were determined by double differential polymerase chain reaction (ddPCR). Expression of ErbB1 (63 patients) and ErbB-2 (171 patients) was assessed by tissue microarray immunohistochemistry (TMA-IHC) and by IHC-based HercepTest, respectively. Disease free survival (DFS) and overall survival (OS) were computed by the Kaplan-Meier method. Univariate and multivariate survival analysis was performed with log rank test and Cox proportional hazard model.

Results: ErbB-1 amplifications and deletions were found in 15% and 31% of cases, respectively, and ErbB-2 amplifications and deletions – in 26% and 3% of cases, respectively. Deletions of ErbB-1 occurred more frequently in node negative ($p = 0.03$) and in PgR negative cases ($p = 0.06$), whereas ErbB-2 AGCN was not related to major clinicopathological characteristics. Overexpression of ErbB-1 and ErbB-2 occurred in 17% and 18% of patients, respectively and both abnormalities were correlated with negative estrogen receptor status ($p = 0.007$ and $p = 0.02$, respectively). ErbB-1 was correlated with lymph node metastases ($p = 0.06$) and larger tumor size ($p = 0.027$). The correlation between expression and AGCN was strong for ErbB-2 ($p = 0.0003$) and insignificant for ErbB-1. ErbB-1 amplification was associated with shorter DFS and OS ($p = 0.03$ and 0.02, respectively) and overexpression – with shorter DFS ($p = 0.04$). ErbB-2 overexpression was associated with shorter OS ($p = 0.02$), whereas prognostic impact of ErbB-2 AGCN did not reach statistical significance. There was a strong correlation between AGCN of ErbB-1 and ErbB-2 ($p = 0.000036$). Patients with co-amplification of both genes tended to be node-positive, but small number of this subset did not allow for statistical analysis.

Conclusions: Overexpression of both ErbB-1 and ErbB-2, and amplification of ErbB-1 carry adverse prognosis in breast cancer patients. Strong correlation between ErbB-1 and ErbB-2 AGCN may indicate an important role of ErbB heterodimers in tumor progression. Clinical relevance of these findings warrant further studies.

314 Poster

VEGF-D in assocation with VEGFR-3 promotes nodal metastasis in human invasive lobular breast cancer

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The aim of this study was to investigate the role of lymphangiogenesis in lymphatic dissemination in invasive lobular breast cancer by examining peri- and intratumoral lymph vessel density as well as the expression of VEGF-C, VEGF-D and VEGFR-3 in these tumors.

By performing immunohistochemistry stainings on human invasive lobular breast cancer tissue samples we assessed the expression of vascular endothelial growth factor C (VEGF-C) and vascular endothelial growth factor D (VEGF-D) in breast cancer cells and the density of lymph vessels and vascular endothelial growth factor receptor 3 (VEGFR-3) vessels in and around the tumor.

We found a significant correlation between peritumoral lymph vessel density and the presence of lymph node metastases ($P = 0.001$). Lymph vessel density also correlated with the number of metastatic lymph nodes ($P < 0.001$). Furthermore a significant correlation was detected between tumor cell VEGF-D expression and lymph node status ($P = 0.001$).VEGF-D expression also correlated with the density of LYVE-1 positive vessels ($P = 0.035$). Tumors positive for both VEGFR-3 and VEGF-D or both VEGFR-3 and VEGF-C had a significantly higher number of metatstatic lymph nodes than tumors with other staining patterns ($P < 0.001$). Finally, tumors that were neither VEGF-D nor VEGFR-3 positive had a lower density of LYVE-1 positive vessels compared with the tumors with other staining patterns ($P = 0.033$).

Our study represents the first simultaneous analysis of VEGF-C/-D expression with LYVE-1, CD34 and VEGFR-3 vessel densities in breast cancer. The results indicate that peritumoral lymph vessel density is associated with lymph node metastases in invasive lobular breast cancer. Moreover, these findings show, that invasive lobular cancer producing VEGF-D, surrounded by VEGFR-3 positive vessels, have a significantly higher peritumoral lymph vessel density as well as a higher number of metastatic lymph nodes.

315 Poster

Real time RT-PCR detection of disseminated tumour cells in bone marrow has superior prognostic significance in comparison with circulating tumour cells in patients with breast cancer

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Purpose: This study assessed the feasibility of using real time RT-PCR analysis to detect disseminated epithelial cells (DEC) in peripheral blood (PB) and bone marrow (BM) of patients with breast cancer (BC). Detection of DEC in BM is an obvious choice in BC, but blood sampling is more