Increased numbers of endothelial progenitor cells in peripheral blood and tumour specimens in non small cell lung cancer patients. A methodological challenge and ongoing debate on the clinical relevance

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Background: Preclinical studies demonstrated that bone marrow derived endothelial progenitor cells (EPC) play an important role in neovascularisation and tumour growth. However, the clinical relevance of EPC on blood vessel formation in NSCLC is unclear. EPC represent an immature subset of CD34 positive cells expressing the glykosylated polypeptide CD133. Early EPC, which are found in the bone marrow, are characterised by their expression of CD34, CD133 and VEGFR2 (KDR). More mature EPC are found in peripheral blood and are positive for VEGFR2 and CD34, but have lost CD133. EPC numbers in the circulation are very low and therefore their detection is technically challenging. In the present study two different immunocytological techniques are presented and compared with each other. In addition, EPC numbers were correlated with plasma levels of VEGF, which is known to be the major trigger to mobilize EPC. Furthermore, the distribution and frequency of CD133 positive EPC in fresh frozen tumour specimens were defined.

Method: 10 NSCLC patients and 5 control patients were analysed in the present study. Blood analyses were done before and after surgery. EPC were isolated from whole blood by using magnetic beads coupled to an antibody directed against CD34 (MACS). After the positive selection, CD34 MNC were divided into two groups. One group underwent FACS analyses labelled with CD34, CD133, VEGFR2, CD45 and VE-Cadherin. The other group was assessed by immunocytological staining for CD133 (MZA). Cryostat sections were stained for CD133, CD31 and Cytokeratin A7. Serum levels of VEGF were quantified by sandwich ELISA.

Results: Compared to the control group, in NSCLC patients, EPC counts were significantly elevated in peripheral blood before and after surgery. The presented alternative method, the MZA assay, correlated statistically significantly with the standard method, the FACS analysis for CD34 VEGFR2 double positive cells. The major advantage of MZA was that even singular positive cells were detectable. Taken pre- and post-surgery measurements together, a statistically relevant/significant trend between EPC numbers and VEGF levels was detected. In seven of ten tumour samples, increased numbers of CD133 positive cells were found and in some cases capillary forming CD133 positive structures were detectable. All EPC counts measured in the tumour patients were statistically significantly elevated compared with the healthy control group.

Conclusions: A significant increase of EPC was detected in patients with NSCLC, suggesting an involvement of EPC in tumour vasculo genesis and tumour growth in these patients. The MZA method is a valuable alternative for the detection of EPC compared to the FACS analysis in the peripheral blood, due to its ability to measure even singular positive cells.

Genomic profile of invasion in lung adenocarcinoma with bronchioloalveolar features (AWBF)

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Background: Bronchioloalveolar carcinoma (BAC) is a subtype of lung adenocarcinoma (ADC) that by definition lacks evidence of stromal, vascular or pleural invasion. However, the histological criteria for diagnosis, their interpretation and the pre-invasive concept of BAC remain controversial. We used array comparative genomic hybridization (CGH) to compare the genome-wide changes of BAC between no or possible micro-invasive samples with invasive AWBF samples to identify candidate genetic markers for invasion or poor prognosis.

Methods: Genomic profiles of 14 non- or micro-invasive BAC and 15 invasive AWBF were obtained using the whole genome submegabase resolution tiling set (SMRT) CGH arrays. Candidate marker genes for invasion were selected using multiple computational tools. Findings were validated in silico, by fluorescent in situ hybridization (FISH) and by quantitative polymerase chain reaction (qPCR) using independent lung adenocarcinoma samples.

Results: Genomic profiles of non- and micro-invasive BAC cannot be distinguished, both showing mainly low copy gains on 1p, 2q, 5p, 7p, 11p, 11q, 12q, 16p, 16q, 17q, 20q and 21q. Most aberrations occurred at the sub-telomeric chromosomal regions. AWBF had a similar pattern of chromosomal aberrations but with greater variability, frequency and range. In addition, deletions were more evident mainly on 3p, 4q, 5q, 6q and 13q. Synchronous BAC and invasive AWBF from the same patient showed increased genomic instability in the latter. Progression was also noted between the BAC-like and invasive areas of AWBF. We identified 113 candidate marker genes for tumor invasion and progression and validated in silico one quarter of them as poor prognostic markers in early stage ADC. We further validated the amplification and overexpression of two genes, PDCD6 and TERT, both located on chromosome 5p that showed prominent genomic change. PDCD6 is a novel poor prognostic marker for overall survival (HR=4.94, 95% CI 1.22-8.52, p=0.02) in early stage ADC patients.

Conclusions: Invasive AWBF can be molecularly distinguished from BAC by greater genomic aberrations, which may include markers for invasion and poor prognosis for early lung ADC.
be associated with significant morbidity. This may reflect our current limitations in staging lung cancer, particularly in detecting early or occult metastases. Several recent studies have demonstrated the ability to detect circulating lung tumour cells by RT-PCR using several gene products and the prognostic value of this investigation. There have been few studies exploring the use of IHC to detect circulating tumour cells. In these studies, IHC was found to be inferior to RT-PCR. The aim of this study was to assess the utility of a novel cell separation method called RosetteSep to generate a concentrated pellet of non-hematologic cells from peripheral blood, which would allow for potentially improved detection of circulating tumour cells by cellular morphology and IHC staining for CEA, CK19, TTF1 and Moc1 compared with RT-PCR using mRNA products of the same genes.

Methods: Forty Stage III or IV, previously untreated, NSCLC patients were accrued at consultation at the Vancouver Cancer Centre (VCC). Twenty healthy volunteer subjects with no prior malignancy, lung disease or ongoing infection were recruited by letter and poster and accrued at VCC. Each consented subject had a total of 17 ml of peripheral blood drawn. The first 2 ml were discarded to avoid epithelial cell contamination from the needle prick through the skin and the remaining 15 ml were collected into heparin tubes. CD45 positive cells were depleted using samples incubated with RosetteSep CD45 Depletion Cocktail (StemCell Technologies Inc) to create cell concentrate pellet samples. Half of this sample was used to create a cell block and slices were taken for cellular morphology and IHC using stains for CEA, CK19, TTF1 and Moc1. The second half of this sample was analyzed using RT-PCR to detect the gene products of CEA, CK19, TTF1 and Moc1.

Results: IHC was reported positive if there was at least 30% cytoplasmic staining for CEA, CK19 and Moc1 and at least 30% nuclear staining for TTF1. Cell morphology was used to confirm the presence of malignant cells in the cell block specimen. The results of RT-PCR were reported as a relative quantity of gene expression in each sample. The detection rate of circulating tumour cells in NSCLC patients by RT-PCR was comparable to the detection rates demonstrated in previous studies. The detection rate of circulating tumour cells by IHC was lower than with RT-PCR but the difference was not significant. No false positives were noted in the healthy volunteers.

Conclusions: This novel cell concentration method improved IHC detection of circulating tumour cells from peripheral blood samples of NSCLC patients. Further refinement of this technique may prove that a smaller volume of blood is required for IHC detection of circulating tumour cells, allowing for better clinical applicability of this investigation. This may ultimately improve “staging” of patients to improve selection of patients for appropriate therapies. Finally, the creation of a cell block would allow for tissue banking for future IHC studies in NSCLC.

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A seventy two gene signature and survival in completely-resected non-small-cell lung cancer (NSCLC)

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Background: Current staging methods are imprecise for predicting the outcome of treatment of NSCLC. The goal of this project was to develop a gene expression profile for stage I and II NSCLC that is able to identify patients that have a high risk of disease progression within 2-3 years after initial diagnosis.

Methods: We used whole-genome gene expression microarrays to analyze frozen-tumor samples from 172 patients (pT1&2, N0&1, MO), who had undergone complete surgical resection in 5 European institutions. Randomly generated numbers were used to assign 2/3 of the samples to an algorithm training group with the remaining 1/3 set aside for independent validation. Cox proportional hazards models were used to evaluate the association between the level of expression and patient survival. We used risk scores and nearest centroid analysis to develop a gene-expression model for the prediction of treatment outcome. 10-fold cross validation was used to prevent model over-training.

Results: 72 genes that correlated with survival were identified by analyzing microarray data and risk scores. Based on the expression of these genes, patients in training and validation groups were classified as either high or low risk. Analysis of predicted risk groups revealed significantly different survival distributions for patients in both the training set (p<0.001) and independent validation set (p=0.01). Genes in our prognostic signature are enriched for genes associated with immune response, antigen binding and protein modification/ubiquination.

Conclusions: Our 72-gene signature is closely associated with overall survival of completely-resected NSCLC patients.