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Real-time RT-PCR of CD146 and VE-cadherin mRNA to detect circulating endothelial cells in peripheral blood of patients with breast cancer

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Introduction: Angiogenesis is a fundamental process in tumour growth and metastatic dissemination. The number of circulating endothelial cells (CECs) in peripheral blood (PB) of patients with cancer reflects the amount of proceeding neoangiogenesis and can therefore be used as a surrogate marker to monitor antiangiogenic therapy. The standard quantification method of CECs is currently based on a complex four-color flow cytometrical analysis. However, real-time RT-PCR technology to quantify endothelial cell-specific mRNA in PB samples has been shown to be a promising alternative approach. This study aimed to compare mRNA expression levels of endothelial-cell specific markers (CD146 and VE-cadherin) in PB of healthy volunteers and patients with breast cancer using real-time RT-PCR.

Methods: PB samples have been collected from 18 healthy volunteers and 18 metastatic breast cancer patients using the PAXgene Blood RNA System. RNA was subsequently isolated with the PAXgene Blood RNA isolation kit according to manufacturer's instructions and reverse transcribed into cDNA with random primers. Real-time PCR analysis was performed with primers and TaqMan probes for both CD146 and VE-cadherin mRNA. Ct values were normalised for beta-actin mRNA expression and gene expression levels were calculated relative to a reference sample (RGE).

Results: VE-cadherin mRNA was increased in patients with breast cancer in comparison to healthy volunteers: the median VE-cadherin mRNA expression level in PB of healthy volunteers was 1.20 (range 0.50–4.18); while this was 2.45 (range 0.69–25.80) for patients with breast cancer ($p = 0.040$). However, the difference in CD146 mRNA expression levels between healthy volunteers and patients with breast cancer did not reach statistical significance: the median CD146 mRNA expression level in PB of healthy volunteers was 0.037 (range 0.020–0.058); while this was 0.058 (range 0.013–0.488) for patients with breast cancer ($p = 0.077$). CD146 and VE-cadherin mRNA expression levels were significantly correlated ($r = 0.401$, $p = 0.017$). A cut-off value was determined as the 95th percentile of the RGE values of the healthy volunteers: this value was 0.058 for CD146 and 4.184 for VE-cadherin mRNA. 9 out of 17 patients with breast cancer had a RGE of CD146 above the cut-off value; while for VE-cadherin 7 out of 18 patients with breast cancer had increased RGEs.

Discussion: Our preliminary results suggest that the quantitative evaluation of endothelial cell-specific mRNA by real-time RT-PCR technology could indeed be a promising tool to monitor the efficiency of antiangiogenic therapy in patients with breast cancer but a larger study population and a comparison with flow cytometry is necessary to confirm this. These studies are ongoing.

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Expression of inhibitor of apoptosis proteins and their relationships with clinicopathologic prognostic factors in breast cancer

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Expression of inhibitor of apoptosis proteins (IAPs) and their clinical relevancies have been studied in various malignancies. While Survivin has been studied in breast cancer, little is known about other IAPs. In this study, the expression levels of 7 IAPs (*NAIP, cIAP1, cIAP2, XIAP, survivin, apollon, and livin*) in 117 breast cancer patients were measured using real-time RT-PCR in an attempt to evaluate their clinical relevancies in breast cancer. IAPs were expressed in 94.9–100% of cases except *XIAP* (70.9%) and *livin* (27.4%). While overexpression of *NAIP* was correlated with unfavorable prognostic factors (T2 and nuclear grade III: $P = 0.026$ and 0.050, respectively), overexpression of *cIAP1* (negative node involvement and stage IIa: $P = 0.017$ and 0.025, respectively) and *apollon* (negative node involvement: $P = 0.044$) were correlated with favorable prognostic factors. Overexpression of *survivin* was correlated with not only unfavorable (age < 35, nuclear grade III, histological grade III, negative estrogen receptor expression, and expression of mutated p53: $P = 0.004$, 0.033, 0.003, 0.026, and 0.057, respectively) but also favorable prognostic factors (negative node involvement and stage ≤ IIa: $P = 0.030$ and 0.057, respectively). Collectively, overexpression of IAPs was correlated with not only unfavorable but also favorable prognostic factors. Longer follow-up is necessary to evaluate the correlation with treatment outcome.

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Lipophilin B: a gene preferentially expressed in breast tissue

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Lipophilin B (LPB) is a member of the secretoglobin gene family. In vivo, LPB exists as a complex with mammaglobin A (MGA), another member of the secretoglobin gene family. Since the expression of both LPB and MGA is largely restricted to breast tissue, both these proteins are candidate biomarkers for breast cancer. The aim of this investigation was to compare the expression of LPB in different types of breast tissue such as "normal" breast, benign breast tissue and malignant breast tissue as well as in a range of non-breast tissues. Using RT-PCR, LPB mRNA was detected in 16/24 (66.7%) normal breast tissues, 102/156 (65.4%) primary breast tumors and 6/34 (17.7%) miscellaneous non-breast tissues. Levels of LPB mRNA were significantly higher in the primary breast tumors as compared to both normal breast tissues ($p = 0.025$) and non-breast tissues ($p < 0.0001$). LPB mRNA was expressed at significantly higher levels in low grade breast tumors compared to high grade tumors ($p < 0.001$). In addition, estrogen receptor (ER) positive breast tumors had significantly higher levels of LPB mRNA ($p = 0.023$) compared to ER negative tumors. In the carcinomas, a significant positive correlation was observed between the levels of LPB and MGA mRNA ($r = 0.62$, $p < 0.0001$, $n = 103$). Using real-time RT-PCR, the LPB gene was found to be amplified in 5/8 (62.5%) of the primary breast tumors. We conclude that LPB mRNA is preferentially but not exclusively expressed in breast tissue Because of its preferential expression in breast tissue and its upregulation in breast cancer, LPB should be explored as a potential breast cancer biomarker.

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Tamoxifen activates CYP3A4 and MDR-1 genes through steroid and xenobiotic receptor (SXR) in breast cancer cells

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Cytochrome P450 monooxygenase 3A4 (CYP3A4) is responsible for the metabolism of endogenous steroids, prescribed drugs and xenobiotics. P-glycoprotein, encoded by multidrug resistance 1 (MDR1) gene, functions as an efflux pump transporting substances from inside of the intestinal cells to the lumen to be absorbed or eliminated. Both genes are regulated by steroid and xenobiotic receptor (SXR), a member of nuclear hormone receptor superfamily. Various endogeneous steroids and drugs function as ligands of SXR. Although CYP3A4, MDR1 and SXR are express mainly in the liver and the small intestine, these genes are also expressed in breast cancer cells such as MCF-7 cells. Since tamoxifen (TAM), an antiestrogen used for breast cancer treatment, may be involved in SXR-mediated transcription and is known to be metabolized by CYP3A4 and P-glycoprotein, we investigated the effect of TAM on these SXR targeted genes in breast cancer cell lines. Expression vector encoding the SXR and reporter plasmid CYP3A4-SXRE-LUC or MDR1-SXRE-LUC were cotransfected into MCF-7 and cultured in the presence of TAM. Transient transfection-based reporter gene assays showed TAM activated the SXR-mediated transcription through CYP3A4 and MDR1 promoters in a ligand- and receptor concentration-dependent manner. Semi-quantitative RT-PCR studies revealed that 4-hydroxy TAM activated the expression of CYP3A4 and MDR1 mRNA in MCF-7 cells. Moreover, to investigate if CYP3A4 or MDR1 mRNAs are expressed in other breast cancer cell lines, we performed semi-quantitative RT-PCR studies using MDA-MB231, T47-D, and ZR75–1 cells. It has been reported that SXR is expressed in MDA-MB231 and T47-D cell lines. CYP3A4 mRNA expression was seen in all cell lines examined. High amount of MDR1 mRNA expression was seen in MDA-MB231 and ZR75–1 cell lines but low amount in T47-D cells. These results support the idea that CYP3A4 and/or MDR1 mRNA expression is present only in SXR-positive breast cancer cells.