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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.

These results suggest that TAM induces CYP3A4 and MDR1 gene expression through SXR, which reduces TAM concentration in breast cancer cells. Thus, we propose that the expression of SXR in breast cancer cells could be a potential risk factor, which induces local TAM resistance.

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Expression of cyclooxygenase-2 in breast carcinogenesis and its relation to Her-2/neu and p53 protein expression in invasive ductal carcinoma

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Although cyclooxygenase-2(COX-2) is overexpressed in various malignant tumors including breast cancers, little is known about the contribution of COX-2 in breast carcinogenesis. Recent studies suggest a possible role of HER-2/neu and p53 gene in controlling COX-2 expression. The purpose of this study was to evaluate COX-2 expression in the successive steps of breast carcinogenesis and to determine its correlation with HER-2/neu and p53 expression in invasive ductal carcinoma of the breast. Immunohistochemical staining with anti-COX-2 antibody was performed in normal breast tissue (n = 15), usual ductal hyperplasia (n = 15), ductal carcinoma in situ (n = 30), and invasive ductal carcinoma (n = 99). Expression of COX-2 in invasive ductal carcinoma was correlated with immuno histochemical expression of HER-2/neu and p53 protein as well as clinicopathologic features.

COX-2 expression was found to be progressively elevated along the continuum from normal tissue to invasive ductal carcinoma (p < 0.001). COX-2 expression in invasive ductal carcinoma significantly correlated with tumor size (p < 0.05) and TNM stage (p < 0.05). COX-2 expression also significantly correlated with p53 and HER-2/neu protein expression (p,0.05 and p,0.001).

On multivariate analysis, only TNM stage and elevated COX-2 expression correlated with survival. Our results suggest the COX-2 may be involved in the carcinogenesis of the breast and may be an independent prognostic indicator in patients with invasive ductal carcinoma. HER-2/neu and p53 are likely to be involved in the regulation of COX-2 expression in invasive ductal carcinomas of the breast.

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Control of estrogen receptor by tumor suppressor protein PTEN in breast cancer cells

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Background: Estrogen receptors (ERs) mediate the biological effects of estrogen in mammary cells by binding to estrogen response elements in the promoter region of target genes or through protein-protein interactions. Anti-estrogens such as tamoxifen inhibit the growth of ER- positive breast cancers by reducing the expression of estrogen-regulated genes. Recent studies show that PI3-Kinase/Akt is involved in anti-estrogen –resistance in ER-positive breast cancers. However, tamoxifen-resistant growth of ER-positive tumors remains a significant clinical problem. Here we show that novel tumor suppressor PTEN, anti-PI3-Kinase stimulates expression of ER in ER-positive breast cancer cells, and PTEN is up-regulated by estrogen.

Methods: MCF-7 cells were treated with 17β-estradiol, and PTEN expression was determined by western-blot analysis. To investigate PTEN effects on ERs in breast cancer cells, MCF-7 cells were infected with vector alone (Ad/LacZ) or PTEN viral vector (Ad/PTEN) for 72 hours.

Results: Estadiol strongly induced PTEN expression in MCF-7 cells. Treatment of MCF-7 cells with Ad/PTEN increased significant PTEN level and induced an increase in ER expression.

Conclusion: These results suggest that ER is an important mediator of expression of the tumor suppressor protein PTEN in breast cancer cells, and in contrast ER is a target of PTEN. These studies form the basis for further investigations to improve the anti-tumor effects of tamoxifen against breast cancers.

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Hyperphosphorylation of translational repressor 4E-BP1 as prognostic factor in human breast cancer

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Background: Activation of the PI3K/Akt/mTOR signal transduction pathway is mainly dependent of membrane receptors and contributes to the development and progression of tumors by prevention of apoptosis and deregulation of cell cycle in a broad spectrum of human tumors. mTOR controls the mammalian translation machinery and constitutes a main controller in cell growth.

Methods: We have analyzed 103 human primary breast tumors with a complete immunohistochemistry (IHC) profile including multiple membrane receptors and phosphorylated (p) signaling proteins: HER2/neu, EGFR, p42/44MAPK, Akt, 4E-BP1, eIF4G, p70S6K, S6 and Ki67. Proposed biomarkers were validated in a subset of both frozen and paraffin-embedded breast tumors by Western blot and IHC.

Results: Activation of PI3K/Akt/mTOR signaling cascade was significantly detected in a high proportion of breast tumors (41.9%). Patients with HER2/neu overexpression showed a higher activation of Akt compared to negative (p < 0.001) and levels of pAkt were correlated with its downstream molecules p4E-BP1 (p = 0.001) and pp70S6K (p = 0.05). Interestingly, p4E-BP1 was mainly expressed in poor differentiated tumors (p < 0.001), significantly correlated with tumor size (p < 0.001) and with presence of lymph node metastasis (p = 0.002). Finally, majority of tumors with p4E-BP1 showed an increased rate of loco-regional recurrence (p = 0.002).

Conclusions: In breast cancer, activation of major cellular signaling pathways is partially mediated by overexpression of membrane erbB receptors, but frequently, activation of signaling proteins is not just dependent of these receptor signals. Evaluation of activation of the converging downstream signaling proteins, as 4E-BP1, could be a stronger prognostic indicator regardless upstream oncogenic alterations. In this study, we show that hyperphosphorylation of 4E-BP1 in breast cancer is associated with high grade, tumor size, lymph node metastasis and loco-regional recurrences.

336   Poster
Clinical usefulness of ATBF1-A expression in breast cancer as a prognostic and predictive marker

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Purpose: The AT motif-binding factor 1 (ATBF1) gene was first identified as a suppressor of the alpha-fetoprotein (AFP) gene through its binding to an AT-rich enhancer element of this gene. The gene is located at chromosome 16q22.3-q23.1 where loss of heterozygosity has been observed in various malignant tumors, especially in breast cancer. This led us to hypothesize that there was a link between levels of ATBF1 expression and the metastatic potential of breast cancer and also, therefore, the prognosis of these patients.

Experimental design: In the present study, the level of ATBF1-A mRNA expression was analyzed using quantitative real-time reverse transcriptase-PCR, in 153 female patients with invasive carcinoma of the breast. ATBF1-A protein expression was also determined by immunohistochemistry from available 90 cases of paired tissues. An association was sought between ATBF1-A expression and various clinicopathologic factors.

Results: ATBF1-A mRNA was expressed at significantly higher levels in breast cancer patients with no axillary lymph node involvement, with small tumors and in estrogen receptor positive tumors. By contrast, no relationship was found between ATBF1-A protein expression and any of the other clinicopathologic factors. Patients expressing high levels of ATBF1-A mRNA tended to have a better prognosis than those expressing low levels. Univariate and multivariate prognostic analyses showed that ATBF1-A mRNA expression is an independent prognostic factor for disease-free survival. Additionally, cytoplasmic expression of ATBF1-A protein tended to be seen in the hormone responsive tumor.

Conclusions: In breast cancer, levels of ATBF1-A mRNA may serve as a predictive indicator of lymph node metastasis. ATBF1-A gene expression may have potential both as a marker of endocrine responsiveness and also as a prognostic indicator for breast cancer progression.

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Tamoxifen induced estrogen receptor activity in endocrine resistant breast cancer

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Introduction: ER co-activator, AIB-1, is associated with decreased disease free survival in breast cancer.

Aims: In breast cancer, though most ER positive patients will initially respond to endocrine treatment, many will eventually relapse. Inappropriate