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Two Methods for the Detection of Breast Cancer Cells in Blood Samples

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Background: Disseminated tumor cells (DTCs) in bone marrow and circulating tumor cells (CTCs) in blood are found in patients with epithelial carcinomas (breast cancer) and are often correlated with poor prognosis of the disease.

In current models circulating tumor cells (CTCs) dissolved from the primary tumor are thought to be responsible for the occurrence of metastases.

However, the detection of CTCs is still a technical challenge. In this study, two methods for tumor cell detection of patients' samples are presented (Real-Time-PCR and immunohisto-chemical staining). Both are known methods with a high sensitivity and a spread marker panel.

Materials and Methods: For the implementation of both methods, different breast cancer cell lines have been used (Cama-1, MCF-7; ZR-75−1). For Real-Time-PCR, blood samples of a healthy donors were spiked with different cell counts (0, 10, 100, 1000, 10,000 and 100,000) per ml blood sample. Total RNA was isolated from the samples, reversely transcribed into cDNA and used for TaqMan Real-Time-PCR reaction with probes against CK8, 18 and 19, while 18S was used as reference. Relative Quantification Curves were drawn by Microsoft™ Excel®.

For immunohistochemical staining, cytospins were prepared from spiked blood samples, fixed with acetone, air dried and stained with antibodies against Her2- and Thomsen-Friedenreich-Antigen (CD176). In a second staining step fluorescently labelled secondary antibodies were applied. Nuclei were counterstained with DAPI, TF-Antigen was stained by Cy2 and Her2-Antigen by Cy3. The staining was controlled and documented by an epifluorescence microscope.

Results: The curve of Relative Quantification for MCF-7 and ZR-75−1 cells shows an increasing slope starting from 1000 cells. For the Cama-1 cell line this trend is already seen from 10–100 cells. In ZR-75−1 all three genes analysed reveal this trend, whereas in Cama-1 and MCF-7 cells a strong increase in Relative Quantification is especially seen for CK8 and 18.

In the immunohistochemical staining, the cells were considered as tumour cells if they showed staining with the antibody-combinations used. Stained cells were counted and recovery rates were determined. For ZR-75−1, 17 of 30 cells which were spiked in the blood samples were recovered. For MCF-7, 18 cells were found in average, and for Cama-1 23 cells were located per slide. The recovery rates calculated from these numbers are 56.6% and 60.0% for ZR-75−1 and MCF-7, for Cama-1 the recovery rate reaches 76.6%.

Conclusion: It seems that Cama-1 cells are a better model than MCF-7 and ZR-75−1 for Real-Time PCR quantification of mamma carcinoma tumor cells in blood samples. MCF-7 and ZR-75−1 cells tend to react more likely immunologically with blood cells of the donor (agglutination between blood cells and cancer cells). The Cama-1 cell line shows also advantages in the detection of tumor cells using immunohistochemical staining. Therefore it will be necessary to test both methods on patient samples to proof their benefit.

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MicroRNAs as Emerging Biomarkers for Micrometastasis Detection in Breast Tumors

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Background: Quantitative Real Time PCR (qPCR) is a highly sensitive method commonly used for the detection of circulating tumor cells (CTC) in peripheral blood of patients with malignant breast tumors. The rationale for using microRNAs (miRNA) as potential therapeutic targets is explained by the fact that miRNAs overexpression in cancer cells has a pathogenic effect. miRNAs may constitute a promising new class of cancer biomarkers for CTC detection. Our primary purpose is to use this approach to identify microRNAs with diagnostic and prognostic value in peripheral blood of patients suffering from breast cancer (BC).

Material and Methods: We used different online bioinformatics tools allowing us to select a panel of microRNAs with high expression in breast tumors while low or no expression in control peripheral blood (PB) and bone marrow. The usefulness of potential up-regulated miRNAs from previous bioinformatic analysis was validated in human breast cell lines: BT549, MCF7, MDA-MB-231, PM1 and T47D; hematopoietic cell lines: JURKAT, KG1 and K562; human breast total RNA (Ambion®), and healthy blood samples (n = 19). *mirVana miRNA* and *RiboPure Blood* isolation kits (Ambion®) were used. Using qRT-PCR miRNAs tumor-related were amplified. Genex 5.0.1 (MultiD Analyses) was used as qPCR data analysis software.

Results: Among a panel of 12 microRNAs analyzed highlight 5 upregulated in breast tumors: miR-32, miR-200a, miR-200b, and the cluster miR-200c/141 fulfilled the premises of bioinformatic searches. For instance, relative expression of mir-32 and mir-141 was 1.09×10^3 and 1.08×10^3 respectively, higher in T47d cells than in control PB (n = 19).

Conclusions: These results suggest that this bioinformatic approach is an useful high-throughout method to identified BC associated miRNAs. Actually our group is actively involved in the study of selected miRNAs for their potential as markers for CTC detection in peripheral blood of BC patients and age-matched healthy control subjects. Supported by Grants PI06/1541 and PI07/0477 from Fondo de Investigaciones Sanitarias (FIS), Instituto de Salud Carlos III. Cancer research in our laboratory is supported by the 'Fundación do CHU A Coruña'.

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The Expression of RANTES (CCL5) is Inversely Correlated with Lymph Node Involvement in Breast Cancer

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Background: Lymph node involvement is the most important prognostic factor in primary operable breast cancer and is mainly related to tumor size. However, it is unclear whether the host affects the process of lymph node metastasis. The chemokine CCL5 plays a role in chemoattraction and activation of immune cells (Weinberg RA; Nature 2007). Its exact functions in tumor biology are somewhat controversial and still not completely understood. We investigated the correlation between the expression of CCL5 in plasma and the presence of lymph node metastases in primary operable breast cancer.

Material and Methods: We measured preoperative plasma levels of CCL5 in 213 postmenopausal breast cancer patients using the BioPlex© technology and compared the expression of CCL5 by the patients lymph node status (negative versus positive). The association between levels of circulating CCL5 and lymph node status was analyzed using a two way ANOVA, allowing an interaction between tumor size and lymph node involvement.

Results: We included 213 patients; 56% were lymph node negative and 44% lymph node positive. Mean tumor size was 28 mm (range 1−90 mm). Across all patients, mean CCL5 levels were 1239 pg/ml (range 158–6814 pg/ml). When allowing the effect of RANTES to differ between different tumor sizes, there was statistical evidence of an association between RANTES and lymph node status (p = 0.048). More specifically, for small tumors the inverse correlation was highly significant (p = 0.014 at tumor size 20 mm) whereas for large tumors, the difference was not found to be significant (p = 0.790 at tumor size 50 mm).

Conclusions: CCL5 concentration in plasma in postmenopausal women with a small primary operable breast cancer are inversely correlated with lymph node involvement. This finding suggests an effect of the host's immune response on the process of lymph node involvement. Our results are currently being validated on a larger cohort of plasma samples of breast cancer patients.

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Quantitative Assessment of Her-2 Expression of Circulating Tumor Cells in Patients with Metastatic and Non Metastatic Breast Cancer

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Background: Circulating tumor cells (CTC) can provide the basis for a real-time liquid biopsy and may guide the use of targeted therapies. We report on unbiased quantification of Her-2 protein expression of CTC.

Materials and Methods: Her-2 assessment of CTC was performed using the CellSearch® system in 103 metastatic (M1) and 88 non metastatic (M0) breast-cancer patients. Digital images of Cytokeratin-PE, DAPI, CD45-APC, and Her-2-FITC from these samples were stored. Expression of Her-2 on CTC was determined by manual review and an automated

algorithm using Her-2-FITC fluorescence of leukocytes to determine the Her-2-expression threshold in each sample.

Results: Her-2 expression of CTC varied greatly within and between patients compared to Her-2 expression of leukocytes. In M1 patients, a threshold of 75% of Her-2 positive CTC in patients with $\geqslant$5 CTC showed a relatively low discrepancy rate between the primary tumor and CTC Her-2 status. Applying this threshold, 9% of M1 patients with Her-2 negative primary tumors had Her-2 positive CTC status and 29% of M1 patients with Her-2 positive primary tumors had Her-2 negative CTC status. No Her-2 discrepancy was observed between CTC and primary tumor in M0 patients.

Conclusions: Our findings demonstrate the feasibility of real-time quantitative and reproducible assessment of treatment targets on CTC, opening a path towards personalized treatment. Her-2 expression is heterogeneous among CTC within each patient. Overall, M1 patients with Her-2 positive primary tumors exhibited Her-2 negative CTC frequently, whereas discrepancies in Her-2 status were limited in other clinical settings.

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Effects of MTor and Insulin Receptor Inhibition in Tamoxifen Resistant Breast Cancer Cells

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Background: PI3KCA-mutations occur frequently in hormone-receptor positive breast carcinomas and may be targeted using mTOR inhibition. In the present study, we aimed to analyse the effects of mTOR inhibition as well as possible interactions with insulin receptor signalling in Tamoxifen-resistant breast cancer cells.

Material and Methods: MCF7 breast cancer cells, harbouring an activating PIK3Ca mutation (Exon 9 1633G>A), and tamoxifen-resistant MCF7 cells (T-MCF7) were treated with the allosteric mTOR complex 1 (mTORC1) inhibitor Everolimus and the active-site mTORC1/mTORC2 kinase inhibitor PP242. In this setting, the effects of insulin receptor signalling on cell growth, motility and viability were investigated by stimulation with insulin or IGF1 and in the presence of siRNA inhibition of the insulin receptor (IR) and insulin like growth factor 1 receptor (IGF-1R).

Results: T-MCF7 showed elevated level of IR/IGFR expression as well as an activated (phosphorylated) ERK1/2 in contrast to the untreated MCF7. The addition of insulin resulted in an increased signal transduction via AKT and ERK1/2. Simultaneous inhibition of mTORC1/2 through PP242 abolished AKT-phosphorylation and led to a complete cell cycle arrest in G0/G1 as well as a substantial decrease of cell viability in MCF7 and T-MCF7. However, mTORC1-inhibition alone using Everolimus resulted only in a partial G0/G1-arrest which could be reversed by addition of insulin. siRNA inhibition of IR demonstrated an effective reduction of MAPK-signalling in both MCF7 and T-MCF7 while siRNAs against IR or IGF1R resulted in an additional decrease of cell viability.

Conclusions: Inhibition of mTOR-signalling reduced cell viability and proliferation in PIK3CA-mutated breast cancer cells independent of an acquired Tamoxifen resistance. However, our data indicate that IR and IGF1R-conferred cell growth may reduce the effects of isolated mTOR inhibition in tamoxifen-resistant breast cancer cells and that additional targeting of the insulin receptor pathway may prove useful in this setting.

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Study of the Effect of Concurrent Use of Letrozole with Radiotherapy to the Cell Death Mechanisms in the Breast Cancer Cell Line

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Purpose: Studies have shown that hormone receptor-positive tumors have molecular, biological and clinical differences. Using hormonal treatment prolongs survival for the majority of hormone receptor positive breast cancer patients. Postoperative radiotherapy (RT) decreases the risk of locoregional recurrence. Studies show that concurrent use of tamoxifen sensitizes cells to RT and increases RT induced pulmonary fibrosis. Whether letrozole sensitizes breast cancer cells to RT has not been determined with sufficient number of studies. There is a single experimental study of breast cancer cells that revealed increased radiation sensitivity with letrozole. The purpose of this study is to investigate the effect of the concurrent use of letrozole with RT, on cell death in the breast cancer cell lines MCF7, and MCF7aro.

Materials and Methods: In our study, in vitro cell culture methods were used. Aromatase expressing MCF7aro breast cancer cell line was chosen as a model and aromatase non-expressing MCF-7cells were used as control. Letrozole was used with varying doses of 100, 500, 1000 nM, and cells were exposed to letrozole for 24–72–144 hours. Irradiation was performed using a Co-60 source with doses 2−4 Gy. Cell death determination experiments were held 24 hours after RT. Cell death was evaluated by measuring caspase-3 activation in cell-lysates and by cell surface annexin V/propidium iodine (PI) staining detected by flow-cytometry. Beclin expression levels known to elevate in autophagy was determined by western blot, The experiments were done in triplicates.

Results: We evaluated caspase-3 and annexin-V/PI results after 24−72–144 h of incubation with letrozole. There was no significant difference for early apoptosis, late apoptosis and necrosis between the letrozole treated and untreated MCF7 cells. In the aromatase expressing MCF7aro cells, we observed that there was a general reduction in cell death in cells treated with letrozole; with a trend towards apoptosis as a cell death modality rather than necrosis.

Also we observed increased autophagy in the MCF7 cells incubated with letrozole only for 24 h and have received 4 Gy irradiation. There were no differences in Beclin levels when these cells received 2−4 Gy irradiation, and were incubated for 72–144 h. On the other hand MCF-7Aro cells which received 2−4 Gy irradiation and were incubated with letrozole showed increased autophagy in all experimental groups.

Conclusion: In conclusion we observed a general reduction of cell death, in hormone-sensitive, receptor-positive and aromatase enzyme expressing cells, after concurrent use of letrozole and radiotherapy; with apoptosis being the primary cell death modality. This observation also correlates with our findings that autophagy which is primarily a survival mechanism may have also increased in these cells. More extensive studies are needed to be able to evaluate the effects of the concurrent use of letrozole and radiotherapy on tumor cell death.

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Expression of Cancer-testis Antigens in Breast Cancer

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Background: Cancer testis (CT)-antigens predominantly expressed in human germ cell lines, but not in somatic tissues, become activated in different cancer types. Several CT-antigens have been shown as possible prognostic marker and therapeutic target for cancer immunotherapy, although the biological functions in cancer are largely unknown. In this study, we investigated the expression of CT-antigens in breast cancer phenotypes to develop strategy of CT-antigen targeting immunotherapy.

Materials and Methods: Expressions of CT-antigens (i.e. NY-ESO-1, MAGE-A, and MAGE-C1) were characterized by immunohistochemistry (IHC) in 100 patients with primary invasive breast carcinoma. Aldehyde dehydrogenase (ALDH)-1 expression, which have been reported as predictive marker of cancer stem cells in terms of resistance to chemotherapy, were also examined. The IHC findings were statistically analyzed with clinical profiles and prognosis of the patients.

Results: NY-ESO-1, MAGE-A, and MAGE-C1 antigens were expressed in 6%, 15%, and 12% of tumor specimens, respectively. NY-ESO-1 and MAGE-A were preferentially expressed in triple negative ($p < 0.01$) or ER negative breast cancers ($p < 0.05$). ALDH-1 expression was observed in 22% of tumor specimens, and was most prevalent in the triple negative breast cancers ($p < 0.001$). Moreover, 41% of ALDH-1 positive specimens were accompanied with expression of any of CT-antigens, some of which showed concomitant expression of CT-antigens and ALDH-1. There was no significant association between the CT-antigen expressions and clinical prognosis (e.g. OS and RFS) possibly due to small sample size in this study.

Conclusion: CT-antigens were expressed in a large proportion of triple negative- and ALDH-1 positive breast cancer specimens. Because of the limited therapeutic modalities for these phenotypes, significance of CT-antigen expressions should be further studied for beneficial immunotherapy in breast cancer patients.

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Differences in MicroRNA Expression Pattern Predetermine Receptor Phenotypes of Breast Cancer Cells

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Background: Tumor growth is tightly associated with regular shifts in microRNA (miRNA) expression pattern. More than 50% of miRNA genes are located in fragile chromosomal regions that are susceptible to