**Gene Expression of Circulating Tumor Cells in Breast Cancer Patients**

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**Abstract**

**Background:** The diagnostic tools to predict the prognosis in patients suffering from breast cancer (BC) need further improvements. New technological achievements like the gene profiling of circulating tumour cells (CTC) could help identify new prognostic markers in the clinical setting. Furthermore, gene expression patterns of CTC might provide important informations on the mechanisms of tumour cell metastasis.

**Materials and Methods:** We performed multiplex-PCR and multiplex-PCR analyses following immunomagnetic separation of CTC. Peripheral blood (PB) samples of 63 patients with breast cancer of various stages were analyzed and compared to a control group of 14 healthy individuals. After reverse-transcription, we performed multiplex PCR using primers for the genes ga733.3, muc-1 and c-erbB2. Mammaglobin1, spdef and c-erbB2 were analyzed applying realtime-PCR.

**Results:** ga733.2 overexpression was found in 12.7% of breast cancer cases, muc-1 in 15.9%, mgb1 in 9.1% and spdef in 12.1%. In this study, c-erbB2 did not show any significant correlation to BC, possibly due to a highly ambient expression. Besides single gene analyses, gene profiles were additionally evaluated. Highly significant correlations to BC were found in single gene analyses of ga733.2 and muc-1 and in gene profile analyses of ga733.3*muc-1* and GA7 ga733.3*muc-1*_mgb1*_spdef.

**Conclusion:** Our study reveals that the single genes ga733.3, muc-1 and the gene profiles ga733.3*muc-1* and ga733.3*muc-1*_mgb1*_spdef can serve as markers for the detection of CTC in BC. The multigene analyses found highly positive levels in BC patients. Our study indicates that not single gene analyses but subtle patterns of multiple genes lead to rising accuracy and low loss of specificity in detection of breast cancer cases.

**Key words:** Mamma carcinoma, PCR, gene profile

**Abbreviations:** BC = breast cancer; CTC = circulating tumour cells; PB = peripheral blood; c-erbB2 = cellular avian erythroblastosis homologue B2; ga733.2 = major gastrointestinal tumour-associated protein; muc-1 = mucin-1; mgb1 = mammaglobin-typ1; mRNA = messenger RNA; PCR = polymerase chain reaction; RT-PCR = reverse transcriptase polymerase chain reaction; RT = reverse transcriptase; spdef = SAM pointed domain-containing ETS transcription factor; FD = First Diagnosis; RD = Relapse Disease; ABCS = Adnagen breast cancer detect kit; ABCD = Adnagen breast cancer select kit

**Introduction**

Circulating tumour cells (CTC) have been shown to play a major role in breast cancer (BC) tumour biology and may have a prognostic value in patients with metastatic disease [1]. Primary tumour architectural characteristics may define its ability to metastize [2; 45-48]. Weak cell-cell connections lead to the dissemination of tumour cells via blood and lymph vessels [3]. Tumour cells entering the circulation depend on the organ microenvironment in order to be able to colonize tissues and to proliferate [4-7].

Altered gene expression is held responsible for a transformed behaviour of tumour tissue [8-11] and may distinguish CTC from healthy cells [12-13]. However, it remains unclear which genes allow a specific detection of CTC. Furthermore, inter-individual variations in gene expression levels due to certain genetic polymorphism additionally challenge these investigations.

The relatively small amount of CTC in PB of cancer patients led to the development of improved detection systems and cell enrichment [1, 14]. Several approaches of immunomagnetic enrichment of CTC in samples of PB have been investigated [15-17]. Beads coated with monoclonal antibodies (mcAb) against epithelial surface proteins achieve a precise detection and extraction of epithelial and carcinoma cells from PB. Subsequently, enriched CTC can be detected by reverse transcriptase polymerase chain reaction (RT-PCR) of altered marker genes putatively supposed to be tumour predictive. Several investigations have analysed various genetic markers to detect CTC.
A French study showed an over expression of *Mucin 1* (*muc-1*), a gene coding for a polymorphic epithelial surface phosphoprotein. This study included both patients with benign breast disease and advanced BC and indicated a significant correlation between the presence of *muc-1*-positive cells and tumour staging. [18]. These findings were reproduced by Felton et al. showing similar results in patients with advanced disease [19].

**Table 1.** Clinical Staging. 63 breast cancer patients presenting with First Diagnosis (FD) or Relapse Disease (RD) were enrolled in this study. G represents disease staging according to the American Joint Committee on Cancer (AJCC).

| G | 1 | 2 | 3 | X | Σ |
|---|---|---|---|---|---|
| FD (%) | 8% | 50% | 38% | 4% | 100% |
| RD (%) | 0% | 61.5% | 30.8% | 7.7% | 100% |

**Cell Separation**

Venous blood samples were collected in EDTA Vacutainer Tubes (Becton Dickenson, NJ USA) and processed within 2 hours using the immunomagnetic separation technique *Adnagen Breast Cancer Select Kit* (ABCs) ([Adnagen, Langenbagen, Germany]) following the manufacturer’s instructions. This technique is based on an immunological selection of shedded non-haematological cells using antibodies, directed against epithelial and probably malignant surface antigens. Briefly, a combination of antibodies is loaded with magnetic particles and preserved in sodium-acid. Antibodies against ga733.3 and two different antibodies against epitopes of *mucin-1* were used.

Before applying the blood samples, the immunomagnetic beads underwent three washing steps in a 1.5 ml tube using 1 ml PBS (Gibco Invitrogen, Karlsruhe Germany). Each step contained a bead separation in a magnetic bar system (*MPC-S of Dynal*). Subsequently 100 µL of immunomagnetic beads were added to 5 ml of blood samples without any additions. Samples, including beads, were inserted in a tube rotator for 2 hours at 20 rounds per minute (rpm) and room temperature (RT).

Using a larger magnetic bar system (*MPC-L of Dynal, Karlsruhe Germany*), bead-loaded cells were accumulated on the *MPC-L* side. Subsequently, supernatants were removed cautiously and PBS (5 ml) was added. PBS addition, *MPC-L* hold and removing of supernatants were repeated two times. At the end of this step, bead-cell-complexes were resuspended in 1 ml of PBS and inserted in *MPC-S*. Supernatants were removed once more and complexes were resuspended in 200 µL of *ABC5* lysis/binding buffer. Finally, samples were inserted into *MPC-S* again. This time, supernatants, including cell lysates with native mRNA, were saved and stored at -20°C.

**STUDY DESIGN**

Our study included 63 BC patients and 14 healthy controls. Patients ages ranged from 33 to 81 years with a median of 60 years. The age of controls varied between 24 and 61 with a median of 41.5 years. Fifty patients presented with first diagnosis (FD) of BC and 13 had relapse disease (RD) at the time of the PB collection. Disease staging according to the *American Joint Committee on Cancer* (AJCC) is summarized in Table 1. The median time interval between surgical treatment and study enrollment was 2.5 months for FD and 60 months for relapse disease (RD).
Next, samples were washed in series with buffer A, B and tris-HCl and inserted in MPC-5 each time. Supernatants were removed. Washing steps with buffer A and B were processed twice, whereas tris-HCl was applied once.

After the final elimination of supernatants, we resuspended mRNA-bead-complexes in 29.5 µl RNase-free water and incubated them in a thermomixer for 5 minutes at 650 rpm and 50 °C.

**REVERSE TRANSCRIPTION (RT)**

Prepared RT-mix consisted of 4 µl 10x buffer RT, 4 µl dNTPs, 2 µl RT (Qiagen, Hilden Germany) and 0.5 µl RNase inhibitor (Promega, Mannheim Germany) for each sample of 29.5 µl mRNA/bead-complexes. RNase-free water was used as control. Transcription was performed in a thermocycler (Perkin Elmer 9600; 2°C/second) with following program:

- 37 °C for 60 min – reverse transcriptase step
- 93 °C for 5 min – inactivation step
- cDNA suspension was stored on ice.

**MULTIPLEX PCR**

Multiplex PCR (mPCR) was executed using 25 µl of HotStar Taq Mastermix (Qiagen, Hilden Germany), 13 µl distilled water, 4 µl PrimerMix Breast Detect (ABCD) and 8 µl of cDNA suspension, RT-control, water as negative control or positive control (ABCD) respectively. Primermix was a threefold combination of primers for ga733.2 (383 bp), muc-1 (293 bp), c-erbB2 (270 bp) and β-actin (114 bp) as control gene 41. The total volume of 50 µl was inserted in a thermocycler (Perkin Elmer 9600; 2°C/second) and PCR was performed with 2 °C/second according to the following program:

1. 95°C for 15 minutes – Taq activation
2. 94°C for 1 minute
3. 60°C for 1 minute
4. 72°C for 1 minute
5. 72°C for 10 minutes – final elongation
6. 4°C

Steps 2 to 4 were repeated for 35 cycles. Finally, DNA suspensions were stored at -20°C. PCR products were analyzed in bioanalyzer (2100 - bioanalyzer Agilent, CA, USA) via DNA chips (version 25-1000bp). Results were registered according to the fluorescence and via conversion in absolute cDNA concentration (ng/µl). As mentioned previously β-Actin was chosen as housekeeping gene.

**REALTIME-PCR**

The expression of c-erbB2, mgb1 and spdef was determined quantitatively using high throughput real-time RT-PCR applying an ABI PRISM® 7900HT cycler (AMX Bioscience, Toreod Norway). Gene expression was quantified by determination of a threshold cycle number (ct). One common threshold was specified manually within the early exponential phase of fluorescence increase and it was used for all genes within one experiment. 'Transcriptase-minus' and 'water-only' controls were included.

The Her-2/neu status of 84.1% of our patients was determined routinely using immunohistochemistry (antibody against Her-2/neu polyclonal DAKO 1:250). Staining results are classified into four levels according to the manufacturer's recommendation. Highly positive synthesis of Her-2/neu (≥ 2+) was observed in 20 cases of BC (20/53; 37.7%).

**STATISTICAL EVALUATION**

Statistical evaluation was performed using SPSS 11.5 for Windows (Microsoft Corp., SA, USA). Specific amplifications both in mPCR and realtime-PCR were registered in units specified for each gene and each group of patients separately. Results were recorded in dichotomized values. The threshold of dichotomized compilation was predetermined at 0.1 ng/µl for mPCR according to manufacturer's recommendation. Realtime-PCR results with (ct) values below 41 cycles were considered as positive. Afterwards, we searched for correlations between cases of FD, RD and healthy participants. Besides single gene evaluations, these searches also included multiple gene evaluations. Single-positive results were defined as samples that featured positive expressions for one gene, whereas twofold-positive results were defined as samples that featured positive expressions of at least two genes [32]. Correlations were checked either by Pearson's two-sided \( \chi^2 \)-test for dichotomized values or by Pearson's bivariate correlation for values with two thresholds. The level of significance was determined as \( p < 0.05 \).

**RESULTS**

**Expression Levels of ga733.2, muc-1 and c-erbB2 in PB through mPCR**

Positive absolute PCR products of BC patients ranged from 0.1 to 8.4 ng/ml for ga733.3, from 0.12 to 6.3 ng/ml for muc-1 and from 0.13 to 1.1 ng/ml for c-erbB2.
In the population of FD we observed specific amplifications for ga733.3 (5/50; 10%), muc-1 (6/50; 12%) and c-erbB2 (7/50; 14%).

Cases of RD exposed overexpression rates for ga733.3 (3/13; 23.1%), muc-1 (4/13; 30.8%) and c-erbB2 (1/13; 7.7%). Healthy controls showed positive expressions for ga733.3 (1/14; 7.1%), muc-1 (1/14; 7.1%) and c-erbB2 (7/14; 50%) (Fig.1).

Expression levels of mgb-1, spdef and c-erbB2 in PB through RT-PCR

4 samples of FD and 2 cases of RD had neither mgb1 specific nor non-specific transcription and were excluded from further examination. The same lack of transcription was found in 2 cases of FD and 1 case of RD concerning spdef. Hence we continued our investigation including 55 patients (44 FD and 11 RD cases) concerning mgb1 and 58 patients (46 FD and 12 RD cases) concerning spdef. Results of remaining products of patients ranged from 28 cycles to 39.5 cycles for mgb1 and from 29.4 cycles to 40.9 cycles for spdef. Specific signals (< 41 ct) for mgb1 were observed in 2/44 (4.5%) cases of FD and 3/11 (27.3%) cases of RD, whereas this value was 0/14 in healthy controls. spdef was detectable in 5/46 (10.9%) cases of FD, in 2/12 (16.7%) cases of RD and in 0/14 healthy controls, respectively. C-erbB2 expression was found in 11/50 (22%) cases of FD, in 5/13 (38.5%) cases of RD and in 10/14 (71.4%) healthy controls. Results ranged from 39.9 cycles to 33.5 cycles (Fig. 2).

View of multiple-positive cases

The analyses of simultaneous gene expressions of ga733.3, muc-1, spdef and mgb1 revealed 15 cases of BC with a positive expression of at least one of these genes. As results of mgb1 were available in 55 cases, we limited this part of the investigation to this cohort of patients. Nine out of 44 positive cases were found in FD cases (9/44; 20.5%) and 6 out of 11 in RD cases (6/11; 54.5%).

View of simple-positive expression of the gene profile ga733.3* muc-1* mgb1* spdef

The 15 participants of the BC group (15/55; 27.3%) had at least one positive expression of the gene pattern GA7 ga733.3* muc-1* mgb1* spdef. Nine cases (20.5%) belonged to the FD cohort and 6 (54.5%) to RD cohort. Healthy controls also demonstrated positive results in 2 cases (14.3%) (Fig. 3).

View of twofold-positive expression of the gene profile ga733.3* muc-1* mgb1* spdef

Regarding at least two positive gene expressions in the pattern specified above, 10 cases (18.2%) of BC patients and no healthy controls fulfilled this criterion. 6 positive cases were seen in the group of FD (6/44; 13.6%) and 4 in the group of RD (4/44; 36.4%) (Fig. 3).

Correlation of expression results to Her-2/neu level via IHC

Since we did not detect any significant correlation of Her-2/neu status and c-erbB2 mRNA in the results of our mPCR and realtime-PCR analyses, the investigation comparing Her-2/neu to other genes and respective gene profiles did not reach any significance.
DISCUSSION

Today there is a growing interest in the gene characterization of CTC in BC, since it may provide important information on the mechanisms for tumour cell metastasis. We analyzed PB samples of BC patients presenting at our gynaecology department. The goal of our study was to determine marker genes with a high sensitivity for the detection of CTC. For this aim, we selected tools to detect CTC and to amplify DNA of predetermined genes, which are potentially overexpressed in tumour cells. Based on data provided by recent studies we used an immunomagnetic method to select circulating carcinoma cells [33-34]. An adverse effect on gene expression by application of direct immunomagnetic enrichment was excluded [35].

Several studies have described muc-1, c-erbB2 and mgb1 as putative markers in BC. Yet, our non-preselect- ed group of BC patients revealed a markedly lower overall expression of muc-1 as compared to other recent investigations [14-36]. Yet, our results concerning mgb1 match those of Grunewald et al. [37]. Nevertheless higher expression of mgb1 (36.6%) in all stages of BC as compared to healthy controls were described by Lin et al. [38].

Additionally our study investigated ga733.3 and spdef which have not been described commonly in BC cases, but which do possess putative marker characteristics [20, 28-30, 39]. Notably, ga733.3 and spdef showed nearly the same overall frequency of overexpression as the above mentioned cancer-associated marker genes. To the best of our knowledge, investigations of spdef in PB applying RT- and realtime PCR analyses have not been described in the literature, yet. Remarkably, the use of ga733.3 as marker was controversially discussed in recent studies due to its minimal basal expression in hematopoietic cells [21]. ga733.3 transcribes the surface molecule Ep-CAM in epithelial cells and serves as target molecule in immunomagnetic procedures of cell detection [41-42]. Thus, our study includes both Ep-CAM as immunomagnetic target transcript and ga733.3 as investigated gene using mPCR.

Comparisons of controls and BC patient groups revealed an increased frequency of ga733.3 (8/63 vs. 1/14), muc-1 (10/63 vs. 1/14), spdef (7/58 vs. 0/25) and mgb1 (5/55 vs. 0/25). It is notable, that Zhong et al. (1999) demonstrated a high frequency of ga733.3 expression in healthy subjects (40%; 16/40) [20]. However, this study did not include any immunomagnetic processes to eliminate haematogenous cells. Another investigation of Rao et al. (2005) revealed a decrease of ga733.3 expression in CTC as compared to ga733.3 expression in tumour tissues, proposing a mechanism through which cells loose adhesion to enter circulation. Yet, this study did not include control subjects [22].

Muc-1 is described as a widely expressed gene in cells of epithelial origin. The literature shows controversial results regarding muc-1. On the one hand, Raynor et al. (2002) demonstrated that muc-1 transcribes a surface molecule, which was found in human non-malignant cells. Hence, a putative marker feature was denied in this investigation [43]. On the other hand, Felton et al. (2004) verified muc-1 expression in 8 of 9 advanced cases of BC, whereas only 1 of 11 healthy controls showed a positive expression. Moreover, they confirmed the expression of muc-1 in non-malignant peripheral lymphocytes [44]. Our investigation revealed 15.9% positive expression in BC samples but only 7% in healthy controls. Notably, former approaches did not include any immunomagnetic separation method and were performed in restricted numbers of patients. Spdef (SAM pointed domain-containing ETS transcription factor, synonym PDEF) is a transcription factor involved in processes like cell proliferation, differentiation and invasion. It is mainly expressed in tissues with high fraction of epithelial cells. Recently, Ghadersohi et al. (2001) described higher expression of spdef in BC tissue as compared to healthy breast specimens [28]. In line with those results, we did not observe any positive results in the control group.

Most notably, in 2005, Van’t Veer et al. emphasized the importance of gene expression profiling of multiple genes in BC, since single or double gene determination did not show satisfactory accuracy [45]. Here we demonstrate, that method-overlapping (mPCR and realtime-PCR) grouping of 4 genes, ga733.3, muc-1, spdef and mgb1 exposes a positive result in 23.8% of our BC patient population. Fourfold profile (ga733.3*, muc-1*, spdef*, mgb1*) of controls levelled off at 14.3%.

To the best of our knowledge, our study represents the first investigation of fourfold gene profiles reported in the literature.

Taken together, our study shows that the single genes ga733.3, muc-1 and the gene profiles ga733.3* muc-1 and ga733.3*2muc-1*spdef*mgb1* can serve as markers for the detection of CTC in BC. Multigene analyses revealed highly positive levels in BC patients. Our study indicates that not single gene analyses but subtle patterns of multiple genes leads to rising accuracy and low loss of specificity in detection of BC cases.

Yet, further studies are needed to better characterize CTC and to better understand metastatic processes in BC. Prospective studies with a larger patient populations and investigations of other target genes may identify aggressive profiles of BC and may influence therapeutic decisions.

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