Glycosyltransferases as marker genes for the quantitative polymerase chain reaction-based detection of circulating tumour cells from blood samples of patients with breast cancer undergoing adjuvant therapy

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Abstract. Altered glycosylation is a predominant feature of tumour cells; it serves for cell adhesion and detachment, respectively, and facilitates the immune escape of these cells. Therefore changes in the expression of glycosyltransferase genes could help to identify circulating tumour cells (CTCs) in the blood samples of cancer patients using a quantitative polymerase chain reaction (PCR) approach. Blood samples of healthy donors were inoculated with certain numbers of established breast cancer cell line cells, thus creating a model system. These samples were analysed by quantitative PCR for the expression of six different glycosyltransferase genes. The three genes with the best results in the model system were consecutively applied to samples from adjuvant breast cancer patients and of healthy donors. FUT3 and GALNT6 showed the highest increase in relative expression, while GALNT6 and ST3GAL3 were the first to reach statistically significant different ΔCT-values comparing the sample with and without addition of tumour cells. These three genes were applied to patient samples, but did not show any significant results that may suggest the presence of CTCs in the blood. Although the relative expression of some of the glycosyltransferase genes exhibited reasonable results in the model system, their application to breast cancer patient samples will have to be further improved, e.g. by co-analysis of patient blood samples by gold-standard methods.

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Introduction

Glycosylation of proteins is the most important posttranslational modification in eukaryotes. Changes in glycosylation patterns are known to occur during cancer formation and progression (1-3). These changes influence cellular functions including cell adhesion and cell immunogenicity (4,5), and facilitate the generation of remote metastases (1). These effects predominantly occur from changes in gene expression levels of glycosyltransferases (GTs), which are often regulated by oncogenes (6,7). Hence, conclusive studies using cancer cell lines and primary tumours (4) have suggested that the gene expression patterns of glycosyltransferases may serve as a prognostic marker in patients with cancer (8,9).

Circulating tumour cells (CTCs) are cells that dissociate from primary epithelial tumours, circulate through blood stream and lymphatic vessels and are the predominant cause of remote metastasis (10-12). All these abilities can reportedly be regulated by glycosylation and therefore establish a strong association to altered glycosylation (13). It is hypothesized that CTCs show different glycosyltransferase (GT) gene expression levels not only in comparison to normal epithelial cells, but also in comparison to surrounding blood cells. This hypothesis therefore suggests that CTCs may be detected and analyzed by quantitative polymerase chain reaction and in a further step might also open roads towards characterizing CTCs by GT gene expression levels.

The present study first analyzed the gene expression levels of six GT genes including: *N*-acetylgalactosaminyl transferase 6 (GALNT6), *N*-acetylglucosaminyl transferase V (MGAT5B), ST3 β-galactoside α-2,3-sialyltransferase 1 (ST3GAL1), fucosyl transferase 3 (FUT3), ST3 β-galactoside α-2,3-sialyl transferase 3 (ST3GAL3) and ST6 (α-N-acetyl-neuraminyl-2,3-β-galac tosyl-1,3)-N-acetylgalactosaminide α-2,6-sialyltransferase 1 (ST6GALNac1) in blood samples of healthy donors that had been inoculated with different numbers of breast cancer cells.

The studied genes were selected due to the following associations with breast cancer: GALNT6 is involved in the

fibronectin pathway and is responsible for breast tumour development and progression (14); MGAT5B has been reported to function in cell motility and metastasis formation (1,5); ST3GAL1 was found to be increasingly expressed in primary breast carcinoma cells and may be correlated to histological grade (15); FUT3 is an effector of metastasis in hormone receptor dependent breast cancers (16); ST3GAL3 is known to be correlated with tumour size and number of affected lymph nodes (9); and ST6GALNac1 is associated with ductal carcinomas and patients with an elevated expression of ST6GALNac1 have a better prognosis for survival (17).

After establishing a model system, the three best performing genes in the primary blood samples of 20 patients with adjuvant breast cancer were compared to 20 healthy donors. The differences in expression levels of the two groups were analysed and correlated to GT gene expression levels with tumour characteristics.

Materials and methods

Blood samples. All patients enrolled in the present study provided a written informed consent and the research was performed in compliance with the Helsinki Declaration (ethical vote 148-12). A total of 20 ml blood was obtained from 20 patients with adjuvant breast cancer and from healthy donors in EDTA-tubes to prevent early coagulation. As a standard, all samples, from adjuvant breast cancer patients as well as from healthy donors with or without inoculation, were treated in the same manner including the subsequent reverse transcription and quantitative PCR reactions. The blood samples were subjected to density gradient centrifugation (400 x g, 30 min); the buffy-coat solutions were discarded and the harvested cell pellets were washed twice with phosphate-buffered saline (Biochrom GmBH, Berlin, Germany) and centrifugation (250 x g, 10 min). After discarding surplus supernatant, the cell pellets were air-dried and stored at -80˚C until further use.

Cell lines. Cama-1 (HTB-21; mammary gland adenocarcinoma), MCF-7 (57136; mammary gland adenocarcinoma) and ZR-75-1 (CRL-1500; ductal carcinoma) breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and sub-cultured according to the manufacturer's instructions. After a detachment step with trypsin (Biochrom GmBH), the cells were counted with a hematocytometer and added to the blood samples in predefined numbers (10 cells/ml blood, 100 cells/ml blood, 1,000 cells/ml blood).

RNA extraction. Blood cell pellets were thawed and dissolved in 1 ml TRIzol® (Invitrogen Life Technologies, Darmstadt, Germany). For innoculation experiments, the breast cancer cells were subsequently added following TRIzol treatment, in order to prevent immunologic effects. Subsequently, 0.2 ml chloroform (Merck Millipore, Darmstadt, Germany) was added and the suspension was vigorously mixed before centrifugation at 12,000 x g for 15 min at 4˚C. The clear liquid phase was aspired and 1 ml isopropanol (Merck Millipore, Darmstadt, Germany) was added. The solution was stored overnight at -20˚C to precipitate the RNA. On the following day, the solution was centrifuged at 12,000 x g for 10 min at 4˚C, the supernatant was carefully removed and the RNA pellet was washed twice with 75% ethanol (Merck Millipore) by centrifugation at 12,000 x g for 10 min at 4˚C. The RNA-pellet was dried, resolved in 20 μl DEPC-treated water and stored at -20˚C until further use. To control the RNA quantity and quality, the concentration was measured using a Nano-photometer (Implen GmbH, Munich, Germany) and denaturing formaldehyde gel electrophoresis was performed.

Reverse transcription. Reverse transcription was performed using SuperScript III First Strand Synthesis Super Mix (Invitrogen Life Technologies), according to the manufacturer's instructions. A total of 4 μg RNA was used for a single reverse transcription reaction.

Quantitative PCR. For quantitative PCR, TaqMan® Fast Universal PCR Master Mix (Applied Biosystems Life Technologies, Foster City, CA, USA) was used. For each gene, a reaction mix consisting of 10 μl Master Mix, 7 μl H_2O and 1 μl TaqMan Primer (GALNT6: Hs_00200529_m1, MGAT5B: Hs_01586300_m1, ST3GAL1: Hs_00161688_m1, FUT3: Hs_00356857_m1, ST3GAL3: Hs_00544033_m1, ST6GALNac1: Hs_00300842_m1) was prepared and added to 2 μl cDNA in a 96-well plate (Micro Amp® Fast Optical 96-Well Reaction Plate with Barcode; Applied Biosystems Life Technologies), which was then sealed with an adhesive cover and analysed in a 7500 Fast Real Time PCR machine (Applied Biosystems Life Technologies). Each gene was analysed as quadruplicate. The PCR cycles were run as follows: initial denaturation (95˚C for 20 s), followed by 40 cycles with denaturation (3 s at 95˚C) and primer extension (30 s at 60˚C). The fluorescence for each gene was displayed by the SDS 1.3.1 software (Applied Biosystems Life Technologies).

Evaluation. The SDS software performed an automatic calculation of CT-, ΔCT-, ΔΔCT- and relative quantification (RQ) values (18). The resulting files were exported to Microsoft® Excel™ (Microsoft Corporation, Redmond, WA, USA) and graphs were generated. Statistical evaluations were performed by using SPSS version 21.0 (International Business Machines Corporation, Ehningen, Germany). For patient samples and negative control samples, the average RQ value of all 20 samples was calculated including the "not detected"-samples, using "0" as replacement character.

Results

RQ-values of glycosyltransferase genes in blood inoculation experiments. The RQ curves showed that 5/6 analysed GT genes could be used for CTC-detection. Only one gene (MGAT5B) was expressed at low levels such that it was not detected by quantitative PCR. FUT3 and GALNT6 exhibited the highest RQ-values, which indicated that they maybe suitable genes for CTC detection from blood samples. Gene expression levels of ST3GAL3 and ST6GALNac1 also increased from the 10-cells/ml blood sample on, but underperformed in the logarithmic model system. The only gene for which the RQ-values markedly increased from the 100-cells/ml blood on was ST3GAL1 (Fig. 1).

Table I. Statistical comparison of ΔCt values of glycosyltransferase genes between samples without addition of breast cancer cells and samples containing 10, 100, or 1,000 tumour cells.

	P-value					
Δ Ct comparison	GALNT6	MGAT5B	ST3GAL1	FUT3	ST3GAL3	ST6GALNac1
0 vs. 10 cells	0.0023	nd	0.5918	0.553	0.0051	0.097
0 vs. 100 cells	$1.3x10^{-5}$	nd	0.684	0.027	$4.9x10^{-5}$	0.0039
0 vs. 1,000 cells	$3.8x10^{-7}$	nd	0.0013	0.0018	$1.3x10^{-6}$	$1.0x10^{-4}$

P-values for GALNT6 showed statistically significant differences between ΔCt values of 0 vs. 10-cell sample, ST3GAL3 had a borderline significance for this sample. FUT3 and ST6GALNac1 had statistically significant different Δ Ct values from the 100-cell sample, while ST3GAL1 was only significant for the 1000-cell sample. nd, not detected; Ct, cycle threshold.

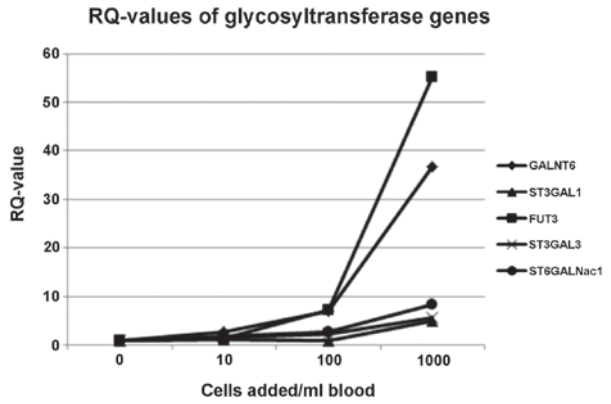

Figure 1. Results of innoculation experiments. Relative quantification (RQ) values for the six glycosyltransferases examined. GALNT6 and FUT3 showed the highest increase in RQ upon increasing the number of breast cancer cells to the blood samples of healthy donors. Expression of MGAT5B was not detected by quantitative polymerase chain reaction.

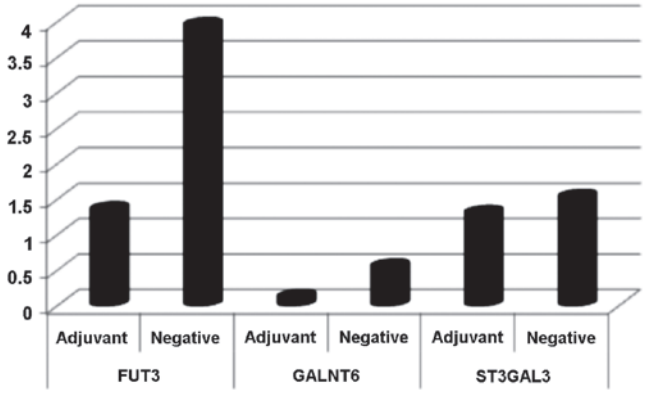

Figure 2. Mean relative quantification (RQ) values of all 20 adjuvant vs. all 20 negative blood samples (from healthy donors) used in the present study. For all three genes examined, the samples from healthy donors showed higher RQ-values as compared with those from breast cancer patients undergoing adjuvant treatment.

Statistical evaluation of inoculation experiments. Increases in gene expression were analysed statistically for each gene and every concentration of added breast cancer cells. ΔCt-values were used for analysis. In a two-tailed T-test, the ΔCt-values of the reference sample without addition of tumour cells were compared to the samples where 10, 100 and 1,000 breast cancer cells had been added. The results of these statistics are shown in Table I. Regarding the statistical evaluation, the best gene for CTC-detection, with significantly different ΔCt-values from the 10-cell level on is GALNT6 (P=0.0023). Accordingly, GALNT6 P-values for the 100- and 1000- cell samples were significantly different from the reference sample and revealed the highest P-values of all examined genes ($P=1.3x10^{-5}$ and $P=3.8x10^{-7}$, respectively). ST3GAL3 and ST6GALNac1 exhibited borderline significantly different ΔCt-values at the 10-cell level (P=0.0051 and P=0.0097), and the values for the 100- and 1,000 cell samples for these two genes were significantly different from the reference sample ($P=4.9x10^{-5}$ and $1.3x10^{-6}$ for ST3GAL3 and 0.0039 and $1x10^{-4}$ for ST6GALNac1). The ΔCt-values of FUT3 showed a significant difference to the 0-cell sample from the 100-cell level (P=0.027 and P=0.0018 for 1000 cells added to the blood sample), whereas the 10-cell sample showed no difference to the sample without addition of tumour cells (P=0.553). ST3GAL1 was only significantly different in ΔCt-value for the 1000-cell sample (P=0.0013). The other two values did not reach significance (P=0.5918 for 10 breast cancer cells added and P=0.684 for 100 breast cancer cells).

RQ-values of adjuvant breast cancer patient samples vs. *negative control samples*. After establishing the model system, GALNT6, FUT3 and ST3GAL3 were selected to be analysed in the patient samples. FUT3 expression was detected in five of the patients and seven of the negative control samples, GALNT6 in 12 and 14 samples, respectively, and ST3GAL3 was detectable in almost all patients (n=19) and negative control (n=17) samples, by quantitative PCR. Average RQ-values for each gene were calculated for the adjuvant breast cancer patients as well as for the negative control group. For the three genes analysed, the average RQ-values were higher overall in the negative control sample group as compared with the patient sample group. The highest average RQ-value divergence was observed in FUT3 (Fig. 2).

Association of RQ-values with tumour characteristics. The RQ-values of gene expression of the adjuvant breast cancer samples were correlated to their tumour characteristics. It was identified that in two patients (No. 6 and 15) all three

Table II. Association of relative quantification (RQ)-values with patient/tumour characteristics.

Patient number	Age (years)	Histological subtype	Tumour size (mm)	Nodal stage	Grading	ER %	PR %	Her2	FUT3	GALNT6	ST3GAL3
1	58	Inv. duct.	1b (11)	1a	2	100	50	pos	n.d.	n.d.	0.025
2	71	Inv. duct. / DCIS	2 (27)	1	2	pos	pos	neg	n.d.	n.d.	0.129
3	50	Inv. duct.	1c (12)	0	3	90	100	neg	n.d.	n.d.	4.185
4	52	Inv. duct.	1c (18)	0	2	90	30	neg	n.d.	n.d.	2.828
5	42	Inv. duct.	3 (58)	0	3	75	0	neg	n.d.	n.d.	n.d.
6	48	Inv. duct.	1c (11)	0	3	0	0	pos	14.893	1.416	5.023
7	42	Inv. duct. / DCIS	1 (1)	1	2	90	1	neg	3.829	0.905	4.327
8	38	Inv. duct.	1b (8)	0	2	100	30	neg	n.d.	n.d.	0.137
9	73	Inv. duct.	1c (12)	1a	2	90	90	neg	n.d.	n.d.	0.041
10	62	Inv. lob. / LIN	2 (38)	0	2	90	80	neg	n.d.	n.d.	0.037
11	69	Inv. lob.	1b (9)	0	2	90	5	neg	1.271	0.210	1.365
12	56	Inv. duct.	1a (4)	0	1	81	5	neg	n.d.	0.018	0.164
13	60	Inv. lob. / DCIS	2 (40)	1a	2	90	30	pos	n.d.	0.043	0.093
14	54	Inv. duct. / DCIS	1a (10)	0	2	80	5	pos	1.481	0.052	0.168
15	58	Inv. duct.	1c (10)	0	2	10	1	neg	5.538	1.194	4.785
16	73	Inv. duct.	2 (25)	0	2	80	80	neg	n.d.	0.110	0.108
17	54	Inv. duct.	1c (13)	0	1	100	100	neg	n.d.	0.213	1.863
18	51	Inv. duct. / lob.	1c (19)	1a	2	90	90	neg	n.d.	0.101	0.082
19	33	Inv. duct.	2 (30)	0	2	99	99	pos	n.d.	0.110	0.100
20	74	Inv. duct.	1c (25)	0	2	99	99	pos	n.d.	0.470	0.499

ER, estrogen receptor positive; PR, progesterone receptor positive; n.d., not detected; pos, positive; neg; negative; inv. duct, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*; lob, invasive lobular carcinoma; LIN, lobular intra-epithelial neoplasia.

gene expression values were upregulated (RQ-values >1). In two further patients (No. 7 and 11), FUT3 and ST3GAL3 were upregulated in comparison to a healthy control sample (Table II). However, significant correlations of changes in gene expressions to age at primary diagnosis, histological subtype, tumour size, nodal state, grading or hormone- and Her-2 receptors, could not be generated from this dataset.

Discussion

Altered glycosylation of membrane bound proteins is a predominant feature of tumour cells that is necessary for cell adhesion and detachment processes and facilitates immune escape of malignant cells. Quantitative PCR-based techniques are already in use for solid tumour profiling and are considered to be objective, robust and cost-effective molecular techniques that could be used in routine cancer diagnostics (19). The present study used a Quantitative PCR assay for the detection of CTCs from peripheral blood. This approach is advantageous for the patient, avoiding painful and sometimes unsatisfactory procedures for biopsies or bone marrow aspirations for analysis.

Glycosyl transferases are a new group of marker genes for CTC-detection from blood samples of patients with breast cancer. In the model system presented, increases in gene expression of different glycosyl transferases were identified through Quantitative PCR, with increasing numbers of breast cancer cells that had been added to the blood sample of a healthy donor.

The inoculation of blood with breast cancer cells identified FUT3 as the most suitable gene for CTC-detection. Further statistical evaluation revealed that GALNT6 could be another valuable marker to detect CTCs, since as few as 10 breast cancer cells per ml blood sample were detectable with statistical significance when compared to the reference sample.

In contrast, in the patient situation, the detection of glycosyltransferase gene expression appears to be more difficult. In some of the patient samples, expressions for the selected genes were not detectable at all. In other cases, the average gene values of the three most promising markers were markedly higher in the 20 negative control samples, as compared to the 20 adjuvant breast cancer samples. It may therefore be concluded that results from the inoculation experiments should be regarded with caution. The model system situation does not necessarily allow conclusions to be drawn in a clinical setting.

To overcome these limitations, a greater patient collective should be analysed by quantitative PCR in order to minimize statistical shortcomings due to low patient numbers. Simultaneously, patient samples could be purified by the CellSearch™ system, which is the only Food and Drug Administration-approved method for CTC-detection from blood samples. The benefit is that especially in the metastatic setting, cleaner cell populations could be analysed and subsequently compared to results of whole blood analysis. Since it is known that the incidence for CTCs is much higher in metastatic patients, we suspect that the establishment of a detection assay based on quantitative PCR with GT markers could be much easier. However, blood samples of metastatic breast cancer patients are more difficult to obtain and thereby sample numbers are limited. Another obstacle is, that a simultaneous CellSearch™ analysis to quantitative PCR is relatively expensive. Importantly, hematopoietic cells display at least a background expression of glycosyltransferases, which emphasizes the role and importance of adequate negative controls of healthy patients.

It could be of major importance to take into account that multiple different subsets of breast cancers exist. In that regard, it may be required to establish standards for each subset (such as hormone-receptor positive, HER2neu overexpression, claudin low, basal, luminal etc.) to balance out the possibility of differential patterns of expression. A prior association between different gradings cannot be ruled out.

Glycosyltransferases may not be the ideal marker genes for quantitative PCR-based detection of CTCs from blood samples of breast cancer patients. This may be due to environmental influence, which can change rapidly according to different handling techniques. Also, precise and careful handling of the primary patient-derived blood samples is critical. Moreover, the monitoring of gene expression of glycosyltransferases cannot be directly associated to aberrant glycosylation and unfolding, as the temporal order of events may differ. However, encouraging results in the present study, especially in the presented model system, suggest that a few select glycosyltransferases could be of use in certain defined situations.

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