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Real-Time qPCR-Based Detection of Circulating Tumor Cells from Blood Samples of Adjuvant Breast Cancer Patients: A Preliminary Study

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Keywords

Circulating tumor cells · RT-qPCR · Cytokeratins · Adjuvant breast cancer

Summary

Background: Circulating tumor cells (CTCs) are cells that detach from a primary tumor, circulate through the blood stream and lymphatic vessels, and are considered to be the main reason for remote metastasis. Due to their origin, tumor cells have different gene expression levels than the surrounding blood cells. Therefore, they might be detectable in blood samples from breast cancer patients by real-time quantitative polymerase chain reaction (RT-qPCR). **Materials and Methods:** Blood samples of healthy donors and adjuvant breast cancer patients were withdrawn and the cell fraction containing white blood cells and tumor cells was enriched by density gradient centrifugation. RNA was isolated and reverse transcribed to cDNA, which was then used in TaqMan real-time PCR against cytokeratin (CK)8, CK18 and CK19. 18S and GAPDH were used as controls. **Results:** All 3 CKs were, on average, found to be significantly higher expressed in adjuvant breast cancer samples compared to negative controls, probably due to the presence of CTCs. Unfortunately, gene expression levels could not be correlated to tumor characteristics. **Conclusions:** RT-qPCR could make up a new approach for the detection of CTCs from blood samples of breast cancer patients, but a correlation of the PCR data to gold standard methods in CTC detection would help to further improve the informative value of the qPCR results.

Introduction

Breast cancer, the neoplasm with the highest incidence worldwide, is the most frequent cause of cancer-related death in women. Fortunately, its mortality has been regressive since the 1970s, but still more than half a million women died from breast cancer and its consequences in 2011.

The main reason for death is seldom the primary tumor itself but the overall tumor mass, due to the occurrence of remote metastases in other organs. The so-called circulating tumor cells (CTCs) are considered to be at the root of metastatic lesions. It is well known that CTCs are cells that dissociate from a primary tumor, move into the circulation through invasion of blood or lymphatic vessels, settle down at secondary sites in the body and can there become the origin of metastasis formation [1–4]. A number of clinical studies were published showing that the presence of CTCs in the blood of cancer patients is correlated with poor outcome of the disease [5–7] and a worse prognosis for overall survival in comparison to patients without CTCs [8–10]. In light of this, CTCs are already used as tumor markers [11].

Different techniques for CTC detection from blood samples have already been established [12]. It has already been shown, that real-time polymerase chain reaction (PCR) is a high-throughput method for detection and molecular characterization of CTCs [13–15], which also allows the monitoring of treatment efficiency [16–19].

The detection of CTCs via real-time quantitative PCR (RT-qPCR) is based on the fact that tumor cells are of epithelial origin, assuming that blood samples with CTCs would show higher expression levels of typical epithelial genes than blood samples without CTCs [20]. Our research group has already worked on the topic of CTC detection from blood samples [21]. We were able to establish and validate this method in an in vitro model system [22, 23]. The present study is now the extension of our former work by

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moving from the model system towards patient samples, focusing on the possibility to detect CTCs in blood samples of adjuvant breast cancer patients to obtain hints on the clinical significance of this method.

For this purpose, blood samples from healthy donors and breast cancer patients in the adjuvant setting were analyzed by RT-qPCR. For that purpose we used standardized gene expression values of cytokeratin (CK)8, CK18, and CK19. We selected these cytokeratin genes as markers since their proteins are also in use as tumor cell markers in the alkaline phosphatase-antialkaline phosphatase (APAAP) staining [24, 25], a routine detection method for tumor cells.

We found an increase in the expression levels of all 3 CK genes in the adjuvant patient group in comparison to the healthy control group, which was statistically significant. Unfortunately, the gene expression values could not be related to tumor characteristics, so that a comparison of the RT-qPCR results with gold standard methods in CTC detection would be necessary to further validate the method.

Materials and Methods

Blood Samples

From each of 20 breast cancer patients, 20 ml blood was withdrawn during primary breast-conserving surgery by using rather fine concave needles, to avoid epithelial cells from the skin to enter the circulation. These patients were treated consecutively with adjuvant breast cancer therapy. Written consent of the patients was obtained prior to the procedure (ethical votum LMU 148–12, in accordance with the Declaration of Helsinki). 20 ml blood was also obtained from each of 20 healthy female donors who had no surgical interventions or previous biopsies before blood withdrawal.

The white blood cell fraction, potentially containing CTCs, was enriched by density gradient centrifugation with Histopaque (Invitrogen, Darmstadt, Germany) for 30 min at 400 × g. Harvested cell pellets were washed twice at 250 × g, 4 °C for 10 min with phosphate-buffered saline (PBS; Biochrom, Berlin, Germany) and stored at –80 °C until further sample processing.

RNA Isolation

RNA was extracted from the cell pellets by the Trizol (Invitrogen, Darmstadt, Germany)/chloroform (Merck, Darmstadt, Germany) extraction method and resuspended in diethylpyrocarbonate (DEPC)-treated water. RNA concentrations and ratios were determined photometrically (Nanodrop, Implen, Munich, Germany), while RNA integrity was controlled by performing denaturing formaldehyde gel electrophoresis.

Reverse Transcription

Of the isolated total RNA, 4 µg was used for cDNA generation by the SuperScript III First Strand Synthesis Super Mix Kit (Invitrogen, Darmstadt, Germany). Reactions were set up and run according to the manufacturer's instructions; thereafter, samples were kept at –20 °C until use in RT-qPCR.

RT-qPCR

RT-qPCR reactions were performed on 96-well plates (Micro Amp® Fast Optical 96-well reaction plate with barcode; ABI, Foster City, CA, USA). For each reaction, 2 µl of the respective cDNA was used, and a reaction mix containing TaqMan® Fast Universal Mastermix (ABI, Foster City, CA, USA), H_2O, and the respective TaqMan® hydrolysis probe (ABI, Foster City, CA, USA; CK8: Hs_02339472_g1, CK18: Hs_01920599_gH, CK19: Hs_00761767_g1, 18S: Hs_03928990_g1, GAPDH: Hs_00266705_g1) was added. Plates were run in

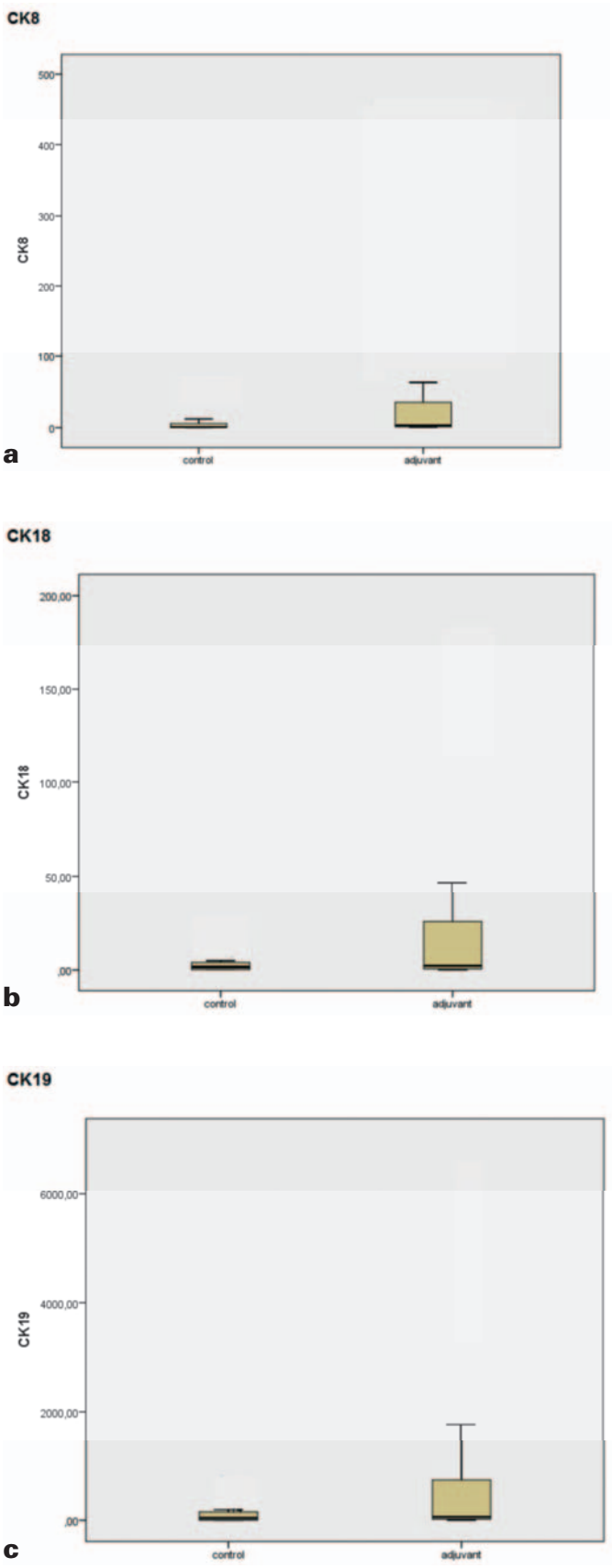

Fig. 1. Comparison of average RQ values from samples of healthy donors versus adjuvant breast cancer samples. (**a**) CK8, (**b**) CK18, (**c**) CK19. In all three cases, the RQ values are higher for the adjuvant breast cancer group than for the negative control group.

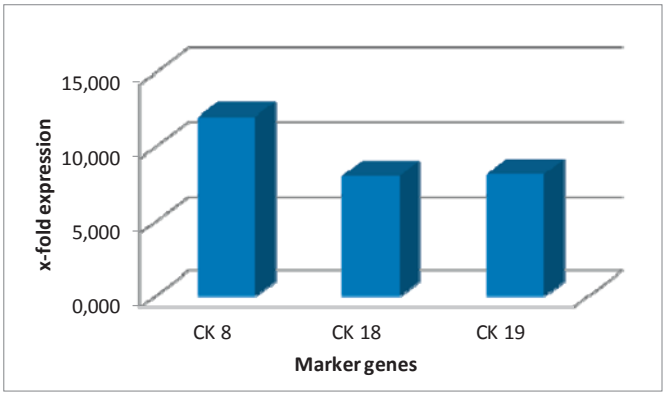

Fig. 2. Fold differences in RQ values between adjuvant breast cancer samples and negative control samples. The most prominent difference is seen for CK8.

Table 1. Average RQ values of all 20 negative controls and adjuvant breast cancer samples

Marker gene	RQ		p
	Negative controls, average (SD)	Adjuvant breast cancer samples, average (SD)	
CK8	4.95 (13.807)	59.607 (128.609)	0.047
CK18	4.151 (6.918)	33.539 (66.957)	0.041
CK19	116.297 (176.702)	952.937 (1826.096)	0.057

For the adjuvant breast cancer samples, the RQ values are significantly higher (indicated by the p-value) than for the negative control samples, indicating the presence of CTCs.
RQ = Relative expression, SD = standard deviation, CK = cytokeratin.

the 7500 Fast Real Time PCR system. 18S (coding for the 18S ribosomal protein) and GAPDH (coding for glyceraldehyde 3-phospate dehydrogenase) were included as reference genes. Reactions for every gene were always set up as quadruplicates including non-template and no-reverse transcription controls; the PCR efficiency of the hydrolysis probes used was stated as 100 ± 10% by the provider, and was therefore not controlled again.

Evaluation

The fluorescence signals were evaluated with the Applied Biosystems Sequence Detection Software (SDS v1.3.1), and CT (cycle threshold), ΔCT, ΔΔCT, and RQ (relative expression) values were automatically calculated according to the $2^{-\Delta\Delta CT}$ method [26]. Non-template and –RT controls did not yield any fluorescent signals.

SDS-generated files were transferred to Microsoft© Excel™ and corresponding graphs were created. Statistical evaluations were done by SPSS v.20, using one-way ANOVA to compare the gene expression levels of sample groups.

Results

Gene expression values of CK8, CK18 and CK19 were standardized to 18S and GAPDH, and average gene expression values were compared between the healthy control group and the adjuvant breast cancer patients (fig. 1a—c, table 1). For CK8, the average RQ value in the control group was 4.95, while in the group of adjuvant breast cancer patients, the value was 59.607. We detected the same trend for CK18 and CK19: For CK18, the averaged value of the healthy control group was 4.151 and the patient group value was 33.539; and for CK19, the average values were 116.297 and 952.937, respectively. This corresponds to a 12-fold difference in gene ex-

Table 2. Correlation of tumor characteristics and RQ values for CK8, CK18, and CK19

No.	T stage	Size, mm	N stage	Histology	Age	ER status	PR status	HER2 status	RQ CK8	RQ CK18	RQ CK19
1	pT1b	11	pN1a (1/2)	inv. duct	58	neg.	neg.	n.d.	7.471	3.272	102.127
2	cT2	27	n.d.	inv. duct	39	n.d.	n.d.	n.d.	3.349	1.528	32.533
3	pT1c	19	pN0 (0/1)	inv. duct	52	neg.	neg.	pos.	0.901	0.32	28.47
4	pT1c	12	pN1a (1/14)	inv. duct	73	pos.	pos.	neg.	3.126	2.639	185.744
5	pT1b	6	pN0 (0/3)	inv. duct	66	pos.	pos.	pos.	0.306	0.185	14
6	pT1c	12	pN0 (0/2)	inv. duct	66	pos.	pos.	neg.	203.192	117.464	3338.293
7	pT2	25	pN0 (0/1)	inv. duct	70	pos.	pos.	pos.	63.171	46.598	1269.884
8	pT1c	13	pN0 (0/2)	inv. duct	70	pos.	pos.	pos.	n.d.	n.d.	n.d.
9	pT2	30	pN0 (0/3)	inv. duct	46	pos.	pos.	pos.	1.432	0.806	21.22
10	pT2	22	pN0 (0/1)	inv. duct	48	pos.	pos.	neg.	5.576	3.424	103.026
11	pT1c	18	pN0 (0/2)	inv. duct	63	pos.	pos.	neg.	0.075	0.054	1.873
12	cT3	55	pN0 (0/2)	inv. duct	53	neg.	neg.	neg.	0.105	0.121	1.259
13	pT1c	12	pN0 (0/2)	inv. duct	52	pos.	pos.	pos.	1.89	1.019	72.961
14	pT2	25	pN0 (0/7)	inv. lob	63	pos.	pos.	pos.	1.629	1.786	52.762
15	pT1c	13	pN0 (0/1)	inv. duct	70	pos.	pos.	pos.	0.407	0.634	17.851
16	pT2	40	pN0 (0/5)	inv. duct	70	pos.	pos.	pos.	155.595	135.514	4635.474
17	pT1c	20	pN0 (0/1)	inv. duct	71	neg.	neg.	neg.	6.834	4.656	106.96
18	pT1c	15	pN0 (0/1)	inv. duct	51	pos.	pos.	neg.	402.414	173.955	6310.456
19	pT1c	18	pN0 (0/4)	inv. duct	50	pos.	neg.	pos.	2.911	2.64	41.437
20	pT1c/pT1b	12/9	pN0 (0/1)	inv. duct	70	pos./pos.	pos./pos.	pos./neg.	273.943	142.751	1769.476

No correlation between tumor data and RQ values could be shown. For patients 3, 5, 11, 12, and 15, a downward deviation of the RQ values from the average RQ value is seen; in patients 6, 7, 16, 18, and 20, the RQ values deviate upwards from the average RQ.
ER = Estrogen receptor, PR = progesterone receptor, HER2 = human epidermal growth factor receptor, RQ = relative expression, CK = cytokeratin, n.d. = not detectable, inv. duct = invasive ductal, inv. lob = invasive lobular, neg. = negative, pos. = positive.

pression for CK8 and an 8-fold difference between the 2 sample groups for CK18 and CK19 (fig. 2).

For statistical analysis, a t-test on independent samples was applied to the data. The test yielded statistically significant differences in gene expression levels for CK8 ($p = 0.047$) and CK18 ($p = 0.041$). For CK19, a borderline significance was detected ($p = 0.057$) (table 1).

Combination of the RQ values of each patient sample and the respective patient and tumor data did not result in any significant correlation (table 2, also for standard deviations).

Discussion

RT-qPCR seems to be a promising method for CTC detection in blood samples [5–7, 13–15] of patients suffering from different types of cancer, such as breast cancer, for example. But still a lot of work needs to be done. As the on-hand preliminary study shows, the presence of tumor cells in blood samples can indeed be detected but the number of CTCs [27] cannot yet be quantified with reference to the used technique. To overcome this limitation, standard curves have to be generated to be able to correlate gene expression levels to the number of CTCs contained in a certain blood sample. However, as different types of breast cancer express different genes at different levels, the creation of reliable standard curves will be a challenging task. Another drawback of the methodology is that it cannot be completely avoided that, by blood withdrawal or previous therapeutical interventions, single epithelial cells will enter the circulation and are later detected as CTCs, as it was shown in a number of publications [28–30], resulting in false-positive outcomes. Although using similar detection methods, other research groups did not find epithelial cells in blood samples of healthy control persons [31, 32]. Therefore it could be generally concluded that sample withdrawal has to be carried out with care, so that the number of such false-positive cells is kept rather small, e.g. by using fine butterfly needles for blood extraction. An analysis of the samples by ancillary methods would help to overcome this obstacle [33].

In the following, a simultaneous analysis of blood samples by RT-qPCR and a gold standard method, the CellSearch™ system, could on the one hand help to generate these standard curves and on the other hand might be useful for the correlation of patient/tumor data and RQ values. Also, an analysis of blood from metastatic patients could improve PCR-based detection of CTCs as the CTC incidence in this patient group is significantly higher, but bears the disadvantage that material from metastatic patients is rather rare. Another important and interesting point would be to decide by the RT-qPCR results whether a certain blood sample came from a cancer patient or was withdrawn from a healthy donor. But for this purpose, a lot of work still needs to be done, especially in defining a set of marker genes on which this decision could be made with high reliability [34–36]. The analysis of more marker genes like Her-2 [37], MMP13 [38], UBE2Q2 [39], or Nectin-4 [40] for their use in CTC detection could help in this point, allowing a more sensitive detection with simultaneous characterization of the tumor cells as well. With the help of such a marker gene panel, an epithelial cell adhesion molecule (EPCAM)-independent CTC detection method could be developed, meaning that the epithelial-to-mesenchymal transition (EMT; [41, 42]) would no longer influence the CTC detection. The latter could be regarded as a drawback of the present study as well. Thereby new roads towards a more individualized treatment with increasing treatment efficiency and reduced therapeutic side effects could eventually be opened.

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Disclosure Statement

The authors declare no conflicts of interest.

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