Quantification of Breast Cancer Cells in Peripheral Blood Samples by Real-Time RT-PCR

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Abstract. *Background: Circulating tumour cells (CTCs) are cells that have detached from a primary tumour, circulate in the peripheral blood, and are considered to be the main root of distant metastases. We present a method for the detection of CTCs by real-time PCR on different cytokeratin markers. Materials and Methods: Blood samples of a healthy donor were mixed with specific numbers of cells from different breast carcinoma cell line cells. RNA was isolated from the samples and transcribed into cDNA. TaqMan real-time PCR for cytokeratins 8, 18 and 19 was carried out and was correlated to that of 18S. Results: Cytokeratin gene expression increased in all samples, when as few as 10 tumour cells were added. In the CAMA-1 cell line, the increase was even greater the more cells were added. Conclusion: By this methodology, cells from mammary carcinoma cell lines can be detected in blood samples. Its benefit will be validated in samples from patients with breast cancer.*

As early as 1869, cells with the morphology of cancer cells had been found by Thomas Ashworth in the blood of a person who died from metastatic cancer (1). Even then he presumed that these cells, today known as circulating tumour cells (CTCs), could play a role in the development of distant metastases. Nowadays, a huge body of literature describes CTCs as cells that, after detaching from the primary tumour and circulating through the blood or lymphatic system, are the main cause of the occurrence of metastases in patients with epithelial cancer (2-4). Numerous studies suggest that detection of CTCs in the blood or disseminated tumour cells (DTCs) in the bone marrow of patients with cancer is linked to poor outcomes of the disease and reduced treatment efficiency (5, 6). There are several different methods for the detection of DTCs and CTCs from bone marrow or blood (7-9). However, withdrawal of bone marrow is a physically painful and exhausting procedure for the patient. The easier access for multiple sampling from peripheral blood makes it the method of choice. Therefore efforts should be made to improve detection efficiencies from these samples. This can be achieved either by cytometric techniques or by nucleic acid-based methods (3). Currently, only one methodology for the discovery and enumeration of CTCs is approved, at least for the metastatic setting by the US Food and Drug Administration (FDA), namely the so-called Cell Search® System (10). This method is based on the immunomagnetic enrichment of CTCs from patient's blood samples, with subsequent staining of these cells. Using this technique, it has been shown that the existence of CTCs is a prognostic factor for overall survival in patients with epithelial cancer types, such as breast, colon or prostate cancer (11-15). In this study, we present a novel nucleic acid-based method for the detection and possible quantification of CTCs utilizing quantitative TaqMan real time PCR. We focused on validating this method in a model system, since this is a necessary step before starting to analyze patient samples. In this regard, we used blood samples from a healthy donor mixed with different numbers of established breast cancer cells (CAMA-1, MCF7, ZR-75-1) and carried out subsequent gene expression analysis for cytokeratins 8, 18 and 19. We selected these three markers in particular, because they are used for histochemical detection of CTCs in so-called APAAP staining (16, 17). The cytokeratin family members are characteristic epithelial cell markers and only weakly expressed in blood cells, rendering them potentially useful for PCR-based detection of CTCs.

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Materials and Methods

Cells. Breast cancer cell lines CAMA-1 (ATCC: HTB-21; mammary gland adenocarcinoma), MCF7 (ATCC: 57136; mammary gland adenocarcinoma) and ZR-75-1 (ATCC: CRL-1500; ductal carcinoma) were used (ATCC, Wesel, Germany). Cell culture was carried out according to international standards, with cells routinely cultivated at sub-confluent states and with re-feeding twice a week. To determine cell numbers, cells were detached, counted in a haemocytometer and diluted with phosphate buffered saline (PBS; Biochrom, Berlin) to the numbers needed.

Blood samples. A healthy individual volunteered to donate 20 ml of blood that was collected into EDTA tubes to prevent coagulation. To remove erythrocytes from the whole blood, Histopaque 1077 (Invitrogen, Darmstadt, Germany) density gradient cell separation was used. Briefly, whole-blood diluted with PBS (1:1) was carefully layered onto histopaque. After a centrifugation step (30 min at 400 ×g) the buffy coat was transferred into a fresh tube. White blood cells were washed twice with PBS and subsequently spun down (10 min at 250 ×*g* and 4˚C). Afterwards, cells were counted and 0, 10, 100, 1,000, 10,000 and 100,000 cells (for CAMA-1, MCF7 or ZR-75-1-alone, and for a mixture of all three cell lines) were added to 1 ml of blood (about 1×10^6 red blood cells). The cells were then centrifuged, the supernatant was removed and cell pellets were frozen at –80˚C.

RNA isolation. To extract RNA, frozen cell pellets were thawed and resuspended in 1 ml of Trizol (Invitrogen, Darmstadt, Germany). After complete resuspension, 0.2 ml chloroform (Merck, Darmstadt, Germany) was added and the solution was vigorously vortexed. After a centrifugation step (15 min at 12,000 ×*g* at 4˚C), the supernatant was transferred into a fresh tube and mixed with 1 ml isopropanol (Merck). The samples were frozen overnight at –20˚C. On the following day, samples were centrifuged again (at 12,000 ×*g* at 4˚C for 10 min), the supernatant was removed and RNA pellets were washed twice with 75% ethanol (Merck). Pellets were then air-dried and resuspended in 20 μl DEPC-treated water. The RNA concentration and ratio were measured photometrically (Nanophotometer™, Implen, Munich, Germany) and only high-quality RNA (absorbance ratio A260/280 between 1.7 and 1.9 guaranteed a high purity of nucleic acids) was used.

Reverse transcription. Four micrograms of RNA in a maximal volume of 6 μl was used for reverse transcription with SuperScript III First Strand Synthesis Super Mix-Kit (Invitrogen). One microliter of provided oligo(dT)-primers and 1 μl annealing buffer was added according to the manufacturer's protocol. Samples were then incubated at 65˚C for 5 min, chilled on ice and 10 μl First-Strand Reaction Mix and 2 μl SuperScript III/RNase Out Enzyme Mix were added before incubation at 50˚C for 50 min. Reverse transcriptase was denatured at 85˚C for 5 min and samples kept at –20˚C until use.

Real-time PCR. First a Mastermix for the target genes (cytokeratins 8, 18 and 19) and reference gene (18S) was prepared. For one reaction, 10 μl TaqMan® Fast Universal PCR Mastermix (ABI, Foster City, CA, USA) was mixed with 1 μl TaqMan primer (ABI; CK8: Hs02339472_g1; CK18: Hs01920599_gH; CK19: Hs 00761767_g1; 18S: Hs 03928990_g1) for the respective gene and 7 μl PCR water. cDNA (2 μl) and 18 μl of the Mastermix were placed into one well of a 96-well plate (Micro Amp® Fast Optical 96-Well Reaction Plate with Barcode; ABI). Each reaction was carried out in quadruplicate. The plate was then sealed with an adhesive cover (ABI) and placed into a 7500 Fast Real-Time PCR system (ABI). To guarantee adequate controls, 18S was used as the reference gene and non-template as well as water–RT controls were implemented. The following cycles were run: 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). Fluorescence for each gene was displayed by the SDS 1.3.1 software (ABI).

Evaluation. Files generated by the SDS Software (CT, ΔCT, ΔΔCT and rq values) were exported to Microsoft® Excel™, and graphs and relative quantification curves (RQC) were drawn. Calculation of relative quantifications values is described in Livak *et al*. 2001 (18). In brief: Average CT value of a gene of interest is related to the 18S average CT value of the same sample. The resulting value is called ΔCT value. In the next step this ΔCT value is set in reference to the ΔCT value of the same gene in the reference sample (here: 0 cells added to blood sample), rendering the so called ΔΔCT value. The formula $2^{-\Delta\Delta CT}$ is then used to calculate relative quantification (rq) values. Rq values >1 show an upregulation of the gene of interest, values <1 mean that the gene is downregulated. Statistical evaluations were made by SPSS® V.20 (IBM®, Ehningen, Germany).

Results

CAMA-1 exhibited relative quantification curves with steady increases of all genes evaluated in a cell number-dependent manner (Figure 1), meaning that, the observed increase in gene expression, is due to the higher number of cells, which express these genes. *CK8*, *CK18* and *CK19* were found to be promising target genes regarding their use as genes for RT-PCR-based detection of CTCs. In detail, CK8 gene expression increased drastically when 1000 or more cancer cells were mixed into the blood sample. CK18 increased in a similar way. However, the RQC for CK18 increased dramatically from 1000 CAMA-1 cells on. The RQC for CK19 follows a different trend: Although an increase in RQ values can be seen for the 10-cell sample (Figure 2), further CK19 expression changed only marginally (Figure 1). Using MCF7 and ZR-75-1 cells, a strong increase of RQ values for all three cytokeratins can be seen comparing control (0 cells) and samples spiked with 10 breast cancer cells (Figures 3 and 4). Further trends for MCF7 and ZR-75-1 spiked samples showed no consistent tendencies. The closest model to the *in vivo* situation seems to be best resembled by using a heterogenous cell pool *in vitro*. We tried to mimic this condition by blending the described cell lines in equal parts and mix the same cell numbers into blood samples. This rendered an RQC with a similar pattern to that for CAMA-1 cells, showing an increase in the expression of all three cytokeratin genes starting with 10 cells (Figure 5). Assuming that only small numbers of cells might be found in the pathological situation of breast cancer, the primary focus of our statistical evaluation was on this range. Between all used

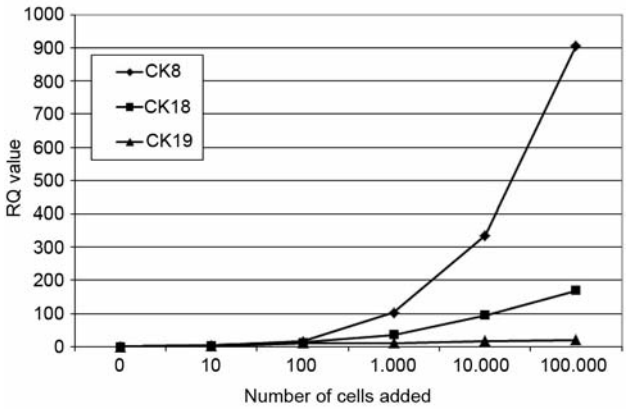

Figure 1. *Relative quantification (RQ) curves for cytokeratins 8, 18 and 19 in CAMA-1 cells. Quantification was relative to that of 18S.*

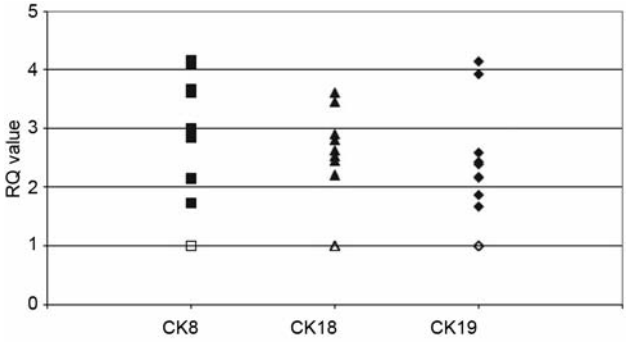

Figure 2. *Comparison of relative quantification (RQ) values of control samples (open symbols) vs. samples spiked with 10 CAMA-1 cells/1×10^6 blood cells (closed symbols).*

single cell lines (CAMA-1, MCF7, ZR-75-1), as well as the cell pool consisting of these cell lines in equal proportions, differences in RQs were statistically significant at *p*-values between 0.011 and 0.012 for the comparison of non-spiked blood samples (negative control sample) *versus* blood samples spiked with 10 cells (Table I).

Discussion

It is generally assumed that the numbers of CTCs in patient blood samples are very small and it is therefore a challenge to detect them (19). For this reason, PCR markers being used to generate calibration curves to detect CTCs have to be as accurate as possible. Out of the three markers investigated in the present study, CK8 and 18 seem to display the required characteristics, especially when a heterogeneous mixture of three established cell lines was used. As few as 10 cancer cells, the first evaluation point in this study, provoked an increase in gene expression recorded in RQCs. This leads to

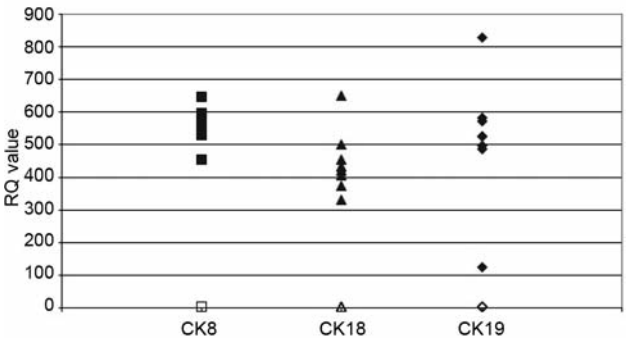

Figure 3. *Comparison of relative quantification (RQ) values of control samples (open symbols) vs. samples spiked with 10 MCF-7 cells/1×10^6 blood cells (closed symbols).*

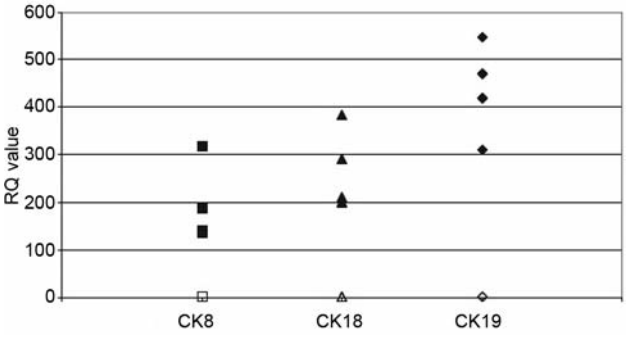

Figure 4. *Comparison of relative quantification (RQ) values of control samples (open symbols) vs. samples spiked with 10 ZR-75-1 cells/1×10^6 blood cells (closed symbols).*

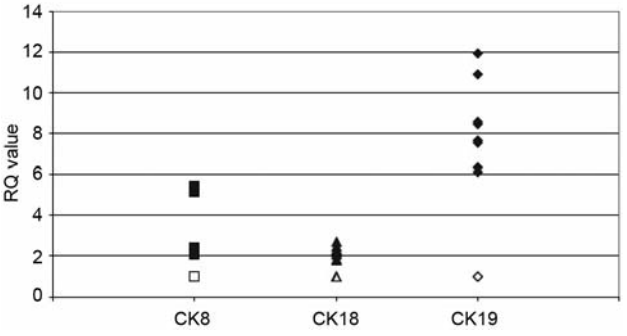

Figure 5. *Comparison of relative quantification (RQ) values of control samples (open symbols) vs. samples spiked with 10 cells of a mixture of the used cancer cell lines/1×10^6 blood cells (closed symbols).*

the assumption that a very low frequency of cells in the peripheral blood, in comparison to the large number of blood cells might be detectable by the presented methodology. This is confirmed by our statistical evaluation showing statistical

Table I. *Statistical significance of differences between relative quantification values for non-spiked and spiked (10 cells) samples. t-test was used for statistical analysis.*

Sample and cytokeratin (nonspiked *vs*. spiked)	*p*-Value
Cell pool CK 8	0.011
CAMA-1 CK 8	0.012
MCF7 CK 8	0.012
ZR-75-1 CK8	0.011
Cell pool CK 18	0.011
CAMA-1 CK 18	0.012
MCF7 CK 18	0.012
ZR-75-1 CK 18	0.011
Cell pool CK 19	0.011
CAMA-1 CK 19	0.012
MCF7 CK 19	0.012
ZR-75-1 CK19	0.011

significances between the negative controls and the samples spiked with 10 cells per millilitre of blood, containing between $1.0\text{-}1.3\times10^6$ surrounding blood cells in all used cell lines and the cell pool. Furthermore, the use of a single cell line seems to be inappropriate for generating a detection model as no constant increase over all samples is displayed for MCF7 and ZR-75-1 cells. This could be due to immunological effects, leading to an agglutination of cancer cells with normal blood cells taking place when high cell numbers are used for spiking. In this study, background variability of the blood sample can be virtually neglected as we only used blood samples of one volunteer donor for all experiments. Furthermore, to ensure that all reverse transcription reactions took place under the same conditions, we performed all reactions at the same time using a standardized protocol. Moreover, sample processing procedures were carried out by the same researcher. However, potential pitfalls have to be taken into consideration. A problem that needs to be mentioned is that adding low numbers of cancer cells to blood is challenging so that probably not all blood samples contain the downright number of cells spiked-in. We tried to minimize these errors by evaluating numerous replicates.

Conclusion

The results of the generated calibration curves for the expression of cytokeratin 8, 18 and 19 genes established in this study shall be transferred to a clinical setting on patient samples to evaluate potential benefits in terms of prediction of outcome or more specifically targeted therapy. Blood samples from patients undergoing adjuvant therapy and with metastatic breast cancer will be compared to samples from a collection of healthy donors (negative controls) by using the same technique as described above. These gene expression levels will be matched to those from the calibration samples with the aim of quantifying the number of CTCs in a given patient sample. As a reference, the results from the established Cell Search® System will be compared to the PCR data. Further putative CTC markers need to be taken into consideration and tested to narrow down non-specific results and increase the chance of detecting and characterizing even the smallest numbers of CTCs. The following might be useful PCR markers in this regard: HER2 (20), insulin-like growth factor-1 receptor (IGF-1) (21), matrix metalloprotease 13 (MMP13)(22-26), ubiquitin-conjugating enzyme family 2Q2 (UBE2Q2) (27), Nectin-4 (28) and aldehyde dehydrogenase (ALDH) (29).

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