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

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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...
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), H2O, 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
the 7500 Fast Real Time PCR system. 18S (coding for the 18S ribosomal protein) and GAPDH (coding for glyceraldehyde 3-phosphate 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−ΔΔCT 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 expression.

<table>
<thead>
<tr>
<th>Marker gene</th>
<th>Negative controls, average (SD)</th>
<th>Adjuvant breast cancer samples, average (SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK8</td>
<td>4.95 (13.807)</td>
<td>59.607 (128.609)</td>
<td>0.047</td>
</tr>
<tr>
<td>CK18</td>
<td>4.151 (6.918)</td>
<td>33.539 (66.957)</td>
<td>0.041</td>
</tr>
<tr>
<td>CK19</td>
<td>116.297 (176.702)</td>
<td>952.937 (1826.096)</td>
<td>0.057</td>
</tr>
</tbody>
</table>

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.

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

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 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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**References**


