Molecular Detection of Telomerase-positive Circulating Epithelial Cells in Metastatic Breast Cancer Patients

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Abstract
The detection of circulating tumor cells and micrometastases may have important therapeutic and prognostic implications. Telomerase is a hallmark of cancer and is absent from normal epithelial cells. The aim of this study was to use telomerase activity as a molecular marker for the detection of cancer cells in blood of patients with breast cancer. Blood samples were collected from 25 women with stage IV breast cancer and 9 healthy volunteers. Peripheral blood mononuclear cells were isolated using ficoll/Hypaque. Immunomagnetic beads coated with an epithelial-specific antibody (BerEP4) were used to harvest epithelial cells from peripheral blood mononuclear cells. Telomerase activity was detected in harvested epithelial cells (HECs) using two different telomerase-PCR-ELISA methods. HECs from blood samples of 21 of 25 (84%) patients with breast cancer were telomerase positive. Telomerase activity was undetectable in HECs from the nine healthy volunteers, demonstrating the specificity of the association between telomerase activity in HECs and stage IV breast cancer. Thus, determination of telomerase activity in HECs appears to be a sensitive, specific, and noninvasive approach for detecting circulating epithelial cancer cells in patients with metastatic breast cancer. This method could be of great value in monitoring the cancer cell proliferation during chemotherapy. This study should be now extended to patients with early-stage breast cancer to investigate the role of telomerase expression by HECs and to evaluate its prognostic value.

Received 12/14/98; revised 3/8/99; accepted 3/8/99.

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Introduction
The sensitive detection of circulating tumor cells and micrometastases may have important therapeutic and prognostic applications. Breast cancer is the most common cause of mortality due to malignant diseases in women, and despite major advances in adjuvant therapy, improvement in survival has been disappointingly small (1). Thus, the detection of circulating tumor cells in the blood may lead to the identification of subgroups of patients who are suitable for more aggressive systemic chemotherapy.

The identification of circulating tumor cells can be accomplished by various techniques, including cytology, flow cytometry, cytogenetics, immunocytochemistry, and immunohistochemistry. However, most of these detection methods have limited sensitivity and specificity (2–4). Detection of circulating epithelial tumor cells can be accomplished by PCR amplification of tumor-specific DNA and mRNA abnormalities that are present in malignant cells. PCR was shown to be more sensitive than conventional techniques for the detection of tumor cells and micrometastases, allowing the identification of 1 tumor cell among 106–107 normal cells (5). However, the major question of its specificity remains unsatisfactory due to the occurrence of normal circulating epithelial cells and illegitimate transcription (4).

Telomerase, a specialized polymerase that directs the synthesis of telomeric repeats toward chromosome ends, is active in ~85% of malignant tumors and is absent from most nonneoplastic tissues and somatic cells (6). Therefore, telomerase is an appealing molecular marker of cancer, and its potential to predict clinical outcome in a range of different neoplasias has been largely documented (7–10). In previous studies, ~75–95% of primary breast cancers showed telomerase activity (11–13), and a recent report has shown that telomerase activity is nearly ubiquitous in invasive breast tumors (14). In addition, careful histological examination and microdissection of pure tumor cells have reduced the number of telomerase-negative breast cancers to 5%.

The aim of this study was to use telomerase activity as a molecular marker for the detection of cancer cells with replicative potential in the blood of patients with breast cancer. Because telomerase activity can be detected at low levels in lymphocytes, epithelial cells were isolated using BerEP4 monoclonal antibody and magnetic beads. Subsequently, telomerase activity was detected by using two distinct telomerase-PCR-ELISA methods.

Materials and Methods
Patients. Twenty-five women, ages 36–78 years, with histologically confirmed stage IV breast cancer were included in this study. Patients were eligible if they had not received any systemic chemotherapy within the 3 weeks prior to blood sampling. Nine healthy female volunteers, ages 23–54 years, were selected as a negative control group.
Blood Samples and Immunomagnetic Separation of Circulating Epithelial Cells. Blood samples (10 ml) were collected in heparinized tubes and stored at 4°C for a maximum of 2 h before experiments. PBMCs were isolated by using Ficoll/Hypaque and resuspended in 1 ml of PBS-2% FCS. Then, 12.5 × 10^6 prewashed immunomagnetic beads covalently coated with the BerEP4 monoclonal antibody (Dynal A.S., Oslo, Norway) were added. The BerEP4 monoclonal antibody recognizes an epitope on the protein moiety of two M, 34,000 and 39,000 glycoproteins expressed at the surface of epithelial cells in normal and malignant tissues and generated from epithelial cell line MCF7 (15). Following incubation at 4°C for 30 min, cells bound to the beads were harvested using a magnetic field. HECs were then washed three times with PBS-2% FCS. Washing efficiency was controlled by microscopic examination to verify that the samples only contained immunomagnetic bead-coated cells. Cells were then stored at −80°C.

Telomerase Assay. HECs were resuspended in 100 μl of lysis buffer (CHAPS) and incubated for 30 min at 4°C. The lysates were centrifuged at 16,000 × g for 20 min at 4°C, and supernatants were transferred into fresh Eppendorf tubes and stored at −80°C until use. Protein concentration was measured in each extract using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA). Telomerase activities were assessed using either the telomerase PCR ELISA kit from Boehringer Mannheim (Mannheim, Germany), the TRAPEZE ELISA Telomerase detection kit (Oncor, Gaithersburg, MD), or both, according to manufacturer’s instructions. Assays have been performed twice in independent experiments on 1 μg of lysates. In the case of negative results and whenever possible (i.e., whenever sufficient amounts of proteins were available), increasing amounts of proteins were tested (up to 10 μg).

In brief, using the telomerase PCR ELISA kit (Boehringer Mannheim), we incubated lysates in the presence of a biotinylated telomerase substrate oligonucleotide (P1-TS primer) at 25°C for 20 min. The extended products were then amplified by a PCR using Taq polymerase, nucleotides, and P2 primers. The PCR conditions were 30 cycles of 94°C for 45 s, 55°C for 30 s, and 72°C for 90 s performed on a PTC-200 thermocycler (M. J. Research, Watertown, MA). Biotinylated TRAP products were incubated for 10 min at room temperature with digoxigenin-labeled probe complementary to telomeric repeat sequence and immobilized onto streptavidin-coated microtiter plates. Afterward, wells were incubated for 30 min at room temperature with anti-digoxigenin-peroxidase-labeled polyclonal antibody. Finally, the amount of TRAP products was determined after the addition of the peroxidase substrate (3,3′,5,5′-tetramethylbenzidine) and the absorbance of each sample was measured at a wavelength of 450 nm (reference wavelength, 595 nm; Microplate Reader 3550, Bio-Rad).

Using the TRAPEZE ELISA telomerase detection kit (Oncor), we incubated lysates for the first steps in presence of a biotinylated telomerase substrate oligonucleotide (b-TS) at 30°C for 30 min. Then, the extended products were amplified by PCR using Taq polymerase (Pharmacia Biotech, Uppsala, Sweden), b-TS, RP primers, and a dideoxynucleotide mix containing dCTP labeled with dinitrophenyl. The PCR conditions were 33 cycles of 94°C for 30 s and 55°C for 30 s. After PCR, the TRAP products were tagged with biotin and DP residues. The labeled products were immobilized onto streptavidin-coated microtiter plates via biotin-streptavidin interaction and then detected by anti-dinitrophenyl antibody conjugated to horseradish peroxidase. The amount of TRAP product was determined after addition of the peroxidase substrate (3,3′,5,5′-tetramethylbenzidine) by measurement of the absorbance at 450 and 595 nm. Each sample was tested along with a heat-inactivated (65°C for 10 min) or RNase-treated aliquot. Reagent controls that lacked cell extracts were also systematically tested.

Determination of the Assay Sensitivity on Serial Dilutions of MCF7 Cells. The epithelial cell line MCF7 was obtained from the American Type Culture Collection (Manassas, VA). In brief, 1000 MCF7 cells were added to 10 ml of heparinized blood from a healthy volunteer. Then, serial dilutions were performed to obtain samples of blood (10 ml) containing decreasing numbers of MCF7 cells (333, 111, 37, 12.3, 4.1, and 1.4 cells respectively). These blood samples with MCF7 cells were subjected to immunomagnetic separation using BerEP4 immunomagnetic beads. Telomerase assays were then performed in triplicates as described earlier. Telomerase activities were then compared to those determined on direct dilutions of MCF7 cells in lysis reagent.

Results

In blood samples taken from 9 healthy volunteers, we did not detect any telomerase activity in circulating epithelial cells harvested using BerEP4 monoclonal antibody, even when telomerase activity was assessed on a fraction of cell extracts equivalent to an initial blood volume of 4000 μl. These data are consistent with the lack of expression of telomerase in normal epithelial cells. To demonstrate the feasibility of the detection of telomerase-positive circulating tumor cells by our method, we used MCF7 breast carcinoma cells as positive controls. We tested telomerase activity in cells recovered from aliquots of whole blood of a healthy donor containing serial dilution of MCF7 cells. We found that our method allowed the detection of as low as 12.3 telomerase-positive cells per 10 ml of blood. The corresponding volume of cell extracts really used for the PCR amplification step theoretically contained 2.3 positive cells. This level of sensitivity was equivalent to that found on direct serial dilutions of MCF7 cell extract.

We, therefore, tested HECs from stage IV breast cancer. We detected telomerase activity in HECs from 13 of 22 (59%) patients using the telomerase PCR ELISA (Boehringer Mannheim) and from 15 of 19 (78.9%) patients using the TRAPEZE ELISA detection kit (Oncor). Taken together, these data show the detection of telomerase activity in HEC from 21 of 24 (84%) patients with metastatic breast cancer (Table 1). RNase treatment or heat inactivation of cell extracts completely eliminated the signals demonstrating the specificity of the enzymatic detection. Furthermore, telomerase-positive samples showed the characteristic processive 6-bp ladder upon PAGE (Fig. 1). The

3The abbreviations used are: PBMC, peripheral blood mononuclear cell; HEC, harvested epithelial cell; TRAP, telomeric repeat amplification protocol.
specific association of telomerase activity with epithelial cells from cancer patients was demonstrated by the absence of contaminating lymphocytes in HEC samples. Indeed, immunofluorescence staining using antibodies against lymphocyte cell surface antigens showed the absence of detectable lymphocytes in HEC samples (data not shown). These data, together with the lack of telomerase activity in normal epithelial cells from healthy volunteers, strongly suggest that telomerase activity reflects the presence of tumor cells in HECs from stage IV breast cancer.

Among telomerase-positive patients, 12 of 21 had detectable telomerase activity when assays were performed on volumes of cell extracts corresponding to 80–500 µl of blood. For 4 of 21 patients, telomerase activity were found with volumes of cell extracts that corresponded to 500-1000 µl of blood. The five remaining patients were only positive for samples that corresponded to volumes of blood greater than 3600 µl. No hint of a relationship between disease status and telomerase activity was observed.

**Discussion**

Telomerase is a potential biomarker for the early detection of cancer (6). Telomerase activity has been examined in most pathological specimens, including fine-needle aspirates, scraping samples, normal secretions (urine and cerebrospinal fluid), pathological fluids (effusions and ascites), aspirates (pancreatic, biliary, and mammary), washes and brushes (oral, colon, bladder, biliary, and lung), biopsies or surgical resected tissues, and frozen tissues. However, to date, telomerase activity had not been used for the detection of circulating cancer cells from solid tumors, except in one brief report about esophageal carcinoma (16). One major problem encountered when blood samples are examined for telomerase activity is the presence of normal hematological cells that express telomerase activity, such as activated lymphocytes (17), although it has been reported that individuals aged >40 years exhibit very low or no detectable telomerase activity in their PBMCs (18). To totally circumvent the telomerase activity from those hematological cells, we separated epithelial circulating cells from PBMCs obtained by density gradient centrifugation (19, 20) using Ber-EP4 antibody-coated magnetic beads. Ber-EP4 does not label hematopoietic cells, and immunomagnetic cell isolation is a simple and reliable method for positive selection of circulating epithelial cells (21).

In this study, we demonstrated that this method associated with telomerase activity assays is sensitive and specific for the detection of tumor cells in the PBMCs of metastatic breast cancer patients. Telomerase has been shown to be a molecular marker of epithelial cancer cells. Thus, compared to other studies in the field (22), telomerase detection in HEC provides a direct detection of tumoral cells. Indeed, the specific association

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**Table 1** Clinical, pathological, and biological status of 25 patients with metastatic breast carcinoma

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age</th>
<th>Menopausal status</th>
<th>Tumor type</th>
<th>Site of metastases</th>
<th>Plasmatic markers&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Treatment (delay since last chemotherapy)</th>
<th>Disease status</th>
<th>Telomerase activity in HECs</th>
<th>Kit used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>Pre</td>
<td>ID</td>
<td>Liver, lung, pleural</td>
<td>1</td>
<td>39</td>
<td>None</td>
<td>PD</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>Pre</td>
<td>ID</td>
<td>Medial lymph nodes</td>
<td>0.76</td>
<td>33</td>
<td>HT</td>
<td>PD</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>Pre</td>
<td>IL</td>
<td>Peritoneum</td>
<td>4</td>
<td>70</td>
<td>CT (3 weeks)</td>
<td>PR</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>Pre</td>
<td>ND</td>
<td>Bone, liver, pleural</td>
<td>2</td>
<td>182</td>
<td>CT (2 weeks)</td>
<td>PD</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>Pre</td>
<td>ID</td>
<td>Sus-clavicular lymph nodes</td>
<td>3</td>
<td>15</td>
<td>CT (3 weeks)</td>
<td>PR</td>
<td>Positive</td>
</tr>
<tr>
<td>6</td>
<td>69</td>
<td>Post</td>
<td>ID</td>
<td>Sus-clavicular lymph nodes, pleural</td>
<td>2</td>
<td>55</td>
<td>CT (4 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>Post</td>
<td>ID</td>
<td>Axillary lymph nodes, skin</td>
<td>8</td>
<td>73</td>
<td>CT (4 weeks)</td>
<td>PD</td>
<td>Positive</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>Pre</td>
<td>ID</td>
<td>Bone, brain, liver</td>
<td>ND</td>
<td>ND</td>
<td>CT (4 weeks)</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>9</td>
<td>53</td>
<td>Post</td>
<td>ID</td>
<td>Bone, liver, pericardial, pleural</td>
<td>30</td>
<td>61</td>
<td>CT (3 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>10</td>
<td>78</td>
<td>Post</td>
<td>ID</td>
<td>Bone, mediastinal lymph nodes, pericardial, pleural</td>
<td>ND</td>
<td>32</td>
<td>HT</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>11</td>
<td>65</td>
<td>Post</td>
<td>ID</td>
<td>Bone, lung, pleural</td>
<td>502</td>
<td>1212</td>
<td>CT (2 weeks)</td>
<td>PD</td>
<td>Positive</td>
</tr>
<tr>
<td>12</td>
<td>45</td>
<td>Pre</td>
<td>ID</td>
<td>Liver, lung, pericardial, skin, sus-clavicular lymph nodes</td>
<td>5</td>
<td>27</td>
<td>CT (3 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>13</td>
<td>47</td>
<td>Pre</td>
<td>ID</td>
<td>Bone, brain, mediastinal lymph nodes</td>
<td>ND</td>
<td>ND</td>
<td>HT, CT (2 weeks)</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>14</td>
<td>66</td>
<td>Post</td>
<td>ID</td>
<td>Liver</td>
<td>2</td>
<td>19</td>
<td>CT (3 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>15</td>
<td>36</td>
<td>Pre</td>
<td>ID</td>
<td>Liver</td>
<td>606</td>
<td>1877</td>
<td>CT (4 weeks)</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>16</td>
<td>53</td>
<td>Post</td>
<td>ID</td>
<td>Brain, liver, skin</td>
<td>ND</td>
<td>483</td>
<td>HT</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>17</td>
<td>42</td>
<td>Pre</td>
<td>ID</td>
<td>Bone, brain, liver, lung</td>
<td>5</td>
<td>35</td>
<td>CT (4 weeks)</td>
<td>PR</td>
<td>Positive</td>
</tr>
<tr>
<td>18</td>
<td>55</td>
<td>Post</td>
<td>ID</td>
<td>Bone</td>
<td>18.6</td>
<td>35</td>
<td>CT (3 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>19</td>
<td>42</td>
<td>Post</td>
<td>ID</td>
<td>Liver</td>
<td>2</td>
<td>23</td>
<td>CT (3 weeks)</td>
<td>PR</td>
<td>Positive</td>
</tr>
<tr>
<td>20</td>
<td>39</td>
<td>Pre</td>
<td>ID</td>
<td>Bone, brain, liver</td>
<td>ND</td>
<td>1796</td>
<td>CT (3 weeks)</td>
<td>PDY</td>
<td>Positive</td>
</tr>
<tr>
<td>21</td>
<td>64</td>
<td>Pre</td>
<td>IL</td>
<td>Skin</td>
<td>21.3</td>
<td>55</td>
<td>CT (4 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>22</td>
<td>46</td>
<td>Pre</td>
<td>ND</td>
<td>Bone</td>
<td>ND</td>
<td>ND</td>
<td>CT (3 weeks)</td>
<td>SD</td>
<td>Positive</td>
</tr>
<tr>
<td>23</td>
<td>44</td>
<td>Pre</td>
<td>ID</td>
<td>Bone, liver, lung</td>
<td>ND</td>
<td>ND</td>
<td>CT (3 weeks)</td>
<td>PD</td>
<td>Positive</td>
</tr>
<tr>
<td>24</td>
<td>67</td>
<td>Post</td>
<td>ID</td>
<td>Liver, lung, mediastinal lymph nodes</td>
<td>ND</td>
<td>1221</td>
<td>HT, CT (4 weeks)</td>
<td>PD</td>
<td>Positive</td>
</tr>
<tr>
<td>25</td>
<td>49</td>
<td>Pre</td>
<td>ID</td>
<td>Bone</td>
<td>ND</td>
<td>245</td>
<td>HT, CT (14 weeks)</td>
<td>PDY</td>
<td>Positive</td>
</tr>
</tbody>
</table>

<sup>a</sup> ID, invasive ductal carcinoma; IL, invasive lobular carcinoma; HT, hormonotherapy; CT, chemotherapy; PD, progressive disease; SD, stable disease; PR, partial response; B, Boehringer Mannheim; O, Oncor; ND, not determined.

<sup>b</sup> Normal values: ACE, <7 ng/ml; CA15-3, <30 units/ml.

<sup>c</sup> Death within 2 months after blood sampling.
of telomerase activity with the presence of tumor cells in HEC is sustained: (a) by the lack of detectable activity in normal epithelial cells from healthy volunteers and (b) by the absence of contaminating lymphocytes demonstrated by immunofluorescence staining. Using two different assays, we found telomerase activity in a high percentage of patients with metastatic breast cancer. It is important to note that our experimental protocol did not allow a comparative evaluation of these two different telomerase ELISA assays. Circulating tumor epithelial cells in breast cancer patients may be extremely rare (22). Positive results obtained from limited volumes of blood suggest that our method displays a reliable threshold of sensitivity. Indeed, our method could detect telomerase activity in 10-ml blood samples containing 12.3 telomerase-positive MCF7 cells.

Concerning the four negative patients and the four additional patients with low levels of telomerase activity, one possible explanation could be that cancer cells have low telomerase levels or even that some metastatic cancer patients don’t have circulating cancer cells. Another explanation could be the variable expression of BerEP4 in breast cancer cells. This particular issue could be overcome by the combined use of different antiepithelial monoclonal antibodies, such as GA73.3EPICAM, which has already been used by others (22). All of these questions should be addressed in a further study, that could particularly include analysis of BerEP4 expression in the surgical pathology samples of patients.

The sensitive detection of circulating tumor cells and micrometastases may have important therapeutic and prognostic implications for breast cancer. About two-thirds of the patients diagnosed with stage I–III breast cancer are candidates for adjuvant or neoadjuvant chemotherapy (5). The aim of this therapy is to prevent the clinical progression of micrometastases that have seeded outside the breast at the time of diagnosis. Approximately 36% of these individuals will remain free of disease using locoregional therapy alone. Routine adjuvant chemotherapy would subject these patients to an unnecessary and toxic treatment. Thus, detection of circulating tumor cells in patients with breast cancer may help to identify patients who might benefit from adjuvant chemotherapy. Furthermore, this method may be useful for the monitoring of the activity of chemotherapy. A recent report shows that the decline in telomerase activity can be a reliable measure of tumor cell killing by antineoplastic agents in vitro (23). Moreover, it has been observed that induction chemotherapy in pediatric leukemia patients decreased telomerase activity in PBMCs to borderline or undetectable levels, suggesting that telomerase activity may be useful in the management of childhood malignancies (24). The efficacy of purging carcinoma cells from autologous bone marrow or peripheral blood stem cells could be evaluated using this method. It also would be of interest to test blood on clinically disease-free patients many years after therapy of a breast carcinoma to determine whether they still have circulating tumor cells. In addition, this approach can be extended to other epithelial malignancies (such as bladder or colorectal carcinomas) frequently associated with metastases.

Nevertheless, it is important to note that the biological significance of small numbers of tumor cells in the circulation remains to be elucidated. Probably only a minority of tumor cells that gain direct access into the circulation have metastatic potential. Indeed, the majority of tumor cells that enter the bloodstream are killed by mechanical, immunological, and/or unknown mechanisms (5). Because telomerase activity in human breast cancers is associated with a more aggressive tumor phenotype (9), the ability of HECs to express telomerase activity could be indicative of a particular biological behavior. However, only prospective studies testing the correlation between the presence of tumor cells in blood and disease-free survival would demonstrate that this technique might be useful in identifying patients with an increased risk of relapse. Further studies must be undertaken to evaluate the frequency of detectable telomerase activity in HEC from earlier stages of invasive breast cancer and various mammary premalignant diseases. It would be also particularly interesting to compare telomerase expression by HEC and other molecular markers such as p53 mutations and Her/erb2 amplification. Such studies would allow to address the clinical and biological significance of telomerase expression in HEC. Indeed, telomerase expression by HEC may be more than a common cancer biomarker because it is directly related to the proliferative capacities of cells and thus may confer to positive cells a greater ability to generate metastases.

In conclusion, this study shows that telomerase-PCR-ELISA associated with immunobead enrichment for epithelial cells may be a useful approach for the detection of circulating tumor cells in the PBMC of patients with breast cancer. The role of telomerase activity in epithelial circulating cells should be further investigated.

Acknowledgments

We are grateful to the nursing staff of the Service Hérault in the Département de Médecine at Institut Gustave Roussy for their helpful contributions.

References


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