Detection of MUC1-expressing Mammary Carcinoma Cells in the Peripheral Blood of Breast Cancer Patients by Real-Time Polymerase Chain Reaction


ABSTRACT

We have prospectively analyzed blood samples of 122 patients with breast disease for the presence of circulating expressing MUC1 cells before and after treatment. Among them, 28 patients had histologically confirmed benign breast disease (group 1), 34 patients had operable breast cancer (group 2), and 60 patients had advanced breast cancer (group 3). Circulating epithelial cells were isolated with BerEP4-coated immunomagnetic beads. Total RNA was extracted and reverse transcribed before analysis by real-time PCR of a MUC1-specific cDNA sequence.

The sensitivity of the reverse transcription-PCR tested with blood spiked with MCF7 cells was one cell in 5 ml of blood. The immunomagnetic separation step was mandatory to obtain the maximum specificity. Control samples from healthy donors never displayed cycle threshold (Ct) values for MUC1 lower than 38. Circulating cells (Ct, <38) were detected in 3 of 28 (11%) cases in group 1, in 8 of 34 (24%) cases in group 2, and in 27 of 60 cases (45%) in group 3. A semiquantitative estimate of blood-borne cells could be derived from the Ct value when below 32 (the lowest was 28) or by the number of positive aliquots of the same blood sample. Thus, immunomagnetic separation, followed by MUC1-specific RT-PCR, allows the semiquantitative detection of circulating mammary cells. A significant correlation between the presence of MUC1-positive cells and the group of breast tumors was observed. The clinical significance of

INTRODUCTION

Death from breast cancer is mainly caused by the presence of distant metastases. At initial diagnosis >95% of patients with breast carcinoma have no evidence of metastatic disease on clinical, radiological, and biochemical examination. In the process of metastasis, tumor cells are scattered from the original site and spread hematogenously or via lymphatic vessels. The detection of circulating tumor cells in the peripheral blood of breast cancer patients has potential interest for unraveling the process of metastasis. However, such detection requires a specific and highly sensitive assay. The amplification of tumor-specific mRNA sequences by RT-PCR has been recently validated for the diagnosis of Ewing sarcoma (1) and chronic myeloid leukemia (2), where specific fusion transcripts characterize the tumor and might be detected in blood or bone marrow. For some solid tumors of epithelial origin, the use of tumor-specific gene alteration may be relevant. For instance, p53 mutations have been used to detect bladder tumor cells in urine (3) and mutated ras gene to detect cells from colorectal cancer in stools (3) and in lymph nodes (4). However, these single mutations are not found consistently, and, therefore, this technique can be applied only in a limited number of patients, with the genetic alteration characterized on the primary tumor. Alternatively, specific gene expression of tumor cells can be used for detection (5–7). Such a gene has to be expressed in all cancer cells, but not or at very low level, in cells normally present in blood. In the present work, we investigated the analysis of MUC1 gene expression to detect circulating mammary cells in patients with breast cancer. MUC1 is a glycosylated mucin normally expressed in epithelial mammary cells (8). In breast cancer MUC1 expression is variable and is often overexpressed.

For some solid tumors of epithelial origin, the use of tumor-specific cDNA sequence.

MATERIALS AND METHODS

Patients and Cell Lines. We have initially analyzed the expression of the MUC1 gene in the human breast cancer cell line MCF7 [a gift from Dr. J. Soudon (Pharmacell, Paris, France)]

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France), to optimize the sensitivity and the specificity of the assay. To validate the RT-PCR method, we also analyzed serial dilutions of human breast cancer cell lines (MCF7) alone and in 5 ml of blood from healthy donors.

We have analyzed 10-ml blood samples obtained from 122 patients with benign or malignant breast tumors. Patients were categorized into three groups (Table 1). Twenty-eight patients had histologically confirmed benign breast disease (fibroadenoma, cyst, epithelial hyperplasia, papilloma, or mastopathy; group 1) and serve as a reference group, 34 patients had an operable breast cancer (group 2, good prognosis), and 60 patients had an advanced breast cancer [inflammatory, large node invasion (>8 N+), and metastatic breast cancer; group 3, poor prognosis]. None of the patients of group 3 had previously received chemotherapy and/or radiotherapy during the 6 months preceding the analysis of their blood samples. Samples were obtained in all cases, before any treatment and in addition for advanced breast cancer, 6 months or more after the latest treatment (surgery, chemotherapy, or radiotherapy). A second sample was obtained for some patients, either 24 h after surgery or after the first cycle of neoadjuvant or adjuvant chemotherapy.

The distribution of all samples is shown in Table 2. A total of 206 blood samples were thus assayed. The study design was approved by the ethical committee (Faculty of Medicine of Cochin University, Paris, France). All patients have given a written informed consent. All patients were assayed in blind.

**Immunomagnetic Separation of Epithelial Cells.** Blood samples and reconstituted samples were collected on EDTA and immediately processed. Epithelial cells were separated from blood samples using immunomagnetic beads, as described by Denis et al. (5). Briefly, blood cells were washed twice with ice-cold PBS. Magnetic beads covalently coated with BerEP4 monoclonal antibody (Dynabeads; Dynal, Oslo, Norway) were then added (4 × 10^6 beads/ml of blood). After incubation at 4°C for 30 min, cells bound to the beads were retrieved with a permanent magnet (Dynal). The beads were then washed five times with ice-cold PBS. Cells immobilized on the beads were then transferred into a microfuge tube for subsequent RNA extraction.

**RNA Extraction and cDNA Synthesis.** RNA was extracted from cells immobilized on the beads with Trizol, as described by the manufacturer (Life Technologies, Inc., Gaithersburg, MD). Glycogen [20 μg; Boehringer Mannheim, Mannheim, Germany] was added as RNA carrier to optimize the extraction efficiency. Samples were stored at −80°C for up to 8 weeks before the assay.

Reverse transcription of RNA was performed in a final volume of 20 μl containing 200 μM of each nucleotide triphosphate, 6.7 mM MgCl₂, 5 units of RNase inhibitor (Promega, Lyon, France), 5 μM random Hexamer (Pharmacia, Uppsala, Sweden), and 200 units of Mo-MuLV reverse transcriptase (Life Technologies, Inc.); and 14 μl of the extracted RNA or H₂O were added. The samples were incubated at 65°C for 5 min, then 42°C for 30 min.

**PCR Conditions.** PCR amplification was performed in the presence of specific target, doubly labeled fluorogenic probes (Taqman probe) that allow an automated quantification of the amplified products in real-time with the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primers and probes were chosen with the assistance of the Primer Express computer program (PE Applied Biosystems). We conducted BLASTN searches against dbEST and nr to confirm the gene specificity of the chosen nucleotidic sequences.

**Results**

**Table 1** Distribution of patients and samples

<table>
<thead>
<tr>
<th>Patients</th>
<th>Pretreatment samples</th>
<th>Posttreatment samples</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>28</td>
<td>28</td>
<td>7</td>
</tr>
<tr>
<td>Group 2</td>
<td>34</td>
<td>34</td>
<td>23</td>
</tr>
<tr>
<td>Group 3</td>
<td>60</td>
<td>60</td>
<td>54</td>
</tr>
<tr>
<td>All</td>
<td>122</td>
<td>122</td>
<td>84</td>
</tr>
</tbody>
</table>

**Table 2** Histological data of group 1 patients

<table>
<thead>
<tr>
<th>Histology</th>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroadenoma</td>
<td>11</td>
</tr>
<tr>
<td>Macro cysts</td>
<td>2</td>
</tr>
<tr>
<td>Normal mammary gland</td>
<td>1</td>
</tr>
<tr>
<td>Duct ectasia</td>
<td>3</td>
</tr>
<tr>
<td>Epithelial hyperplasia</td>
<td>4</td>
</tr>
<tr>
<td>Papilloma</td>
<td>1</td>
</tr>
<tr>
<td>Phylloides tumor</td>
<td>1</td>
</tr>
<tr>
<td>Fibrocytic mastopathy</td>
<td>5</td>
</tr>
</tbody>
</table>

Each experiment was performed in two independent runs (ARN extraction and reverse transcription), each in duplicate (PCR; finally, each blood sample was assayed in quadruplicate). The assessment of quality control was performed in standardized PCR conditions, including in each experiment a positive control (100 ng of reversed ARN of MCF7 cells) and two negative controls (one with no template and one negative reverse transcription control).
Patients with Breast Cancer (Groups 2 and 3). Patients with breast cancer were divided into two groups according to the stage of the disease. Group 2 (34 patients) included patients with advanced breast cancer (inflammatory disease, more than eight involved nodes, and metastatic disease) treated by chemotherapy. The mean age was 57 years (range, 31–83) and 49 years (range, 28–83) for patients of group 2 and group 3, respectively.

Ten-milliliter blood samples from 34 patients with operable breast cancer (group 2) were collected before surgery and, for 23 of them, also 24 h after surgery or before the second cycle of chemotherapy. Blood samples of 60 patients with advanced breast cancer (group 3) were collected at diagnosis and, for 45 of them, before the second cycle of chemotherapy (Table 2). The data obtained for these patients are presented in Table 4.

In all cases, patients were considered positive if one or more of the quadruplicate PCR assays, before or after treatment, disclosed a Ct ≤37. In these conditions, MUC1 transcripts were detected in 8 of 34 patients of group 2 (24%) and in 27 of 60 patients of group 3 (45%). In the latter group, 15 of 37 (41%) patients with advanced breast cancer and 12 of 23 (52%) patients with metastatic breast disease were positive for MUC1 transcripts (Table 4). The lowest Cts were 31 and 28 in group 2 and group 3, respectively. In group 3, we observed more positive replicates for one sample (Table 5). In addition, nine (15%) patients with advanced breast cancer (group 3) had a positive blood sample either before or after chemotherapy.

If we consider the positivity of only the sample collected before treatment, we observed 18% of positive cases in group 2 and 33% of positive cases in group 3.

A significant correlation between MUC1 positivity and the three groups of patients was observed ($\chi^2$ test, $P < 0.01$).

**DISCUSSION**

RT-PCR of several expressed genes (13, 14) has been used to detect circulating breast cancer cells, assuming a sufficient tumor cell specificity of these genes (carcinembryonic antigen, mammaglobin, and cytokeratin 19), but none has yet proved to be specific or sensitive enough for clinical use. In the present work, we have combined immunomagnetic separation of circulating epithelial cells with the RT-PCR detection of MUC1 transcripts. The MUC1 gene is expressed in breast tumors, with a high, but variable, level of transcripts, but mainly up-regulated (10). The exact role of this overexpression and the regulation of MUC1 expression is not completely understood. Its role in tumor progression is evoked because it has been demonstrated that entire cell membrane expression of MUC1 reduces cell-cell and cell-extracellular matrix interaction (15, 16). MUC1 is mainly expressed in breast and ovarian tissue, and much lower in other epithelial tissues where other Muc genes are mainly expressed. Aside from breast and ovary, MUC1 epitopes are found in cells of the erythroid lineage and in 2–10% of monocellular cells in normal bone marrow (17). Thus, the initial immunomagnetic separation is mandatory to eliminate nonepithelial cells from the samples.

The main issue of the method resides in the balance between high sensitivity and specificity. Real-time detection and quantitation of specific PCR products using Taqman probes is adapted to the specific detection of rare events, because the sensitivity of the assay allowed us to detect 5 pg of RNA. It is highly reproducible, and it eliminates the major risks of contamination encountered with other types of detection (nested PCR and gel electrophoresis; Ref. 18). But, as previously described by others, with all tissue-specific genes tested, we observed a basal level of transcription in normal blood, named illegitimate transcription (19–26). The sequences of these transcripts are identical to tissue-specific mRNAs found in breast. When we performed serial dilutions of mononuclear cells and breast cancer cells, we observed that the level of expression of MUC1 illegitimate transcripts was about 10,000-fold lower than that observed in human breast cell lines, as described by others.
Fig. 1  Expression of Muc1 RNA by real-time PCR. A, amplification curve of serial dilutions of an initial amount of MCF7 RNA: 1 (100 ng), 2 (10 ng), 3 (1 ng), 4 (100 pg), 5 (10 pg), and 6 (5 pg). Cycle number is plotted versus change in normalized reporter signal (ΔRn). Inset, standard curve plot of the log of MCF7 RNA concentration versus Ct. The standard curve shows five orders of linear dynamic range. B, expression of Muc1 RNA of nine blood samples of breast cancer patients. Four are positive (#117, #123, #125, #126), and five are negative and represented by an asterisk (#115, #116, #118, #127, #128). C+ is the positive control: an initial amount of 100 ng of MCF7 RNA and C− is also represented by an asterisk and is a no-template sample, as described in “Materials and Methods.” C, ethidium bromide-stained 2% agarose gel of amplified Muc1 products of the same controls and patients in B. A unique band of 100 bp is observed in positive control and samples.
unclear. The histological diagnosis confirmed that there was no
MUC1 only in one of four replicates, indicating yet a very low level of
(benign breast disease), three patients were positive. In all three
positive cases and the patient group was found. In group 1
involvement. A significant correlation between the frequency of
metastatic breast cancer, and patients with major node
advanced breast tumors (inflammatory breast cancer), patients
of poor prognosis (group 3), comprising patients with locally
b

Table 4 Frequency of positive cases in patients of groups 2 and 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Positive cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 2</td>
<td>34</td>
<td>8 (24)</td>
</tr>
<tr>
<td>Group 3</td>
<td>60</td>
<td>27 (45)</td>
</tr>
<tr>
<td>Advanced breast cancer</td>
<td>37</td>
<td>15 (41)</td>
</tr>
<tr>
<td>Metastatic breast cancer</td>
<td>23</td>
<td>12 (52)</td>
</tr>
<tr>
<td>All groups</td>
<td>94</td>
<td>35 (37)</td>
</tr>
</tbody>
</table>

Table 5 Frequency of positivity in replicates of the same sample in all patients

<table>
<thead>
<tr>
<th>MUC1 positivity</th>
<th>Group 1 (n = 28)</th>
<th>Group 2 (n = 34)</th>
<th>Group 3 (n = 60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>One replicate</td>
<td>3 (11%)</td>
<td>7 (21%)</td>
<td>15 (25%)</td>
</tr>
<tr>
<td>More than one</td>
<td>0</td>
<td>1 (3%)</td>
<td>10 (17%)</td>
</tr>
</tbody>
</table>

(27). In addition, five normal samples of healthy donors were
similarly treated by immunomagnetic separation and RT-PCR, and all displayed a Ct >45 (data not shown). Thus, the specific
detection of mammary cells requires an enrichment of mam-
mary cells, with a contamination of <100 blood mononuclear
cells. Positive immunomagnetic separation reaches this goal.
We used an immunomagnetic separation of epithelial cells with
magnetic beads coated with an epithelial-specific monoclonal antibody (Ber-EP4). The immunohistochemical staining of nine
human breast cancer specimens showed a consistent staining in
tumor cells by Ber-EP4 (28). This antibody has already been
used to enrich in epithelial colorectal cells (5, 29, 30) and breast
cancer cell lines (6) with a good yield. We have previously
obtained a consistent recovery of 80–100% of [3H]thymidine-
labeled MCF7, MDA-MB231, or T47D cells in serial dilutions
in normal blood.4 The combination of immunobead isolation of
epithelial cells with RT-PCR detection of MUC1 mRNA in-
creases the specificity of the method, because no MUC1 tran-
scripts were detected in normal blood samples in these experi-
mental conditions. Real-time PCR allowed a consistent
sensitivity of detection of one cell in 5 ml of blood.

For the clinical evaluation of this method, we defined three
groups of patients. The reference group (group 1) included pa-
tients with benign breast tumors, whereas two groups of breast
cancer patients were analyzed: (a) a group of good prognosis,
patients with operable breast tumors (group 2); and (b) a group
of poor prognosis (group 3), comprising patients with locally
advanced breast tumors (inflammatory breast cancer), patients
with metastatic breast cancer, and patients with major node
involvement. A significant correlation between the frequency of
positive cases and the patient group was found. In group 1
(benign breast disease), three patients were positive. In all three
cases, the Ct was at the threshold of detection and was reached
only in one of four replicates, indicating yet a very low level of
MUC1 transcripts. The clinical significance of this result is
unclear. The histological diagnosis confirmed that there was no
histological feature of malignancy: two patients had a fibroad-
enoMA, and one patient had a fibrocystic mastopathy. False
positive cases may occur with a very highly sensitive detection
method. MUC1 is not specific of malignant cells and is also
expressed in normal and benign breast cells. The detection of
normal mammary cells that could have been mobilized by an
invasive diagnosis, such as core biopsy or fine-needle aspiration
can be eliminated, because only one patient had had a biopsy 4
months before the assay. In addition, none of these cases was
found positive 24 h after surgery. Interestingly, one of these
patients had a tumor occurring on the contralateral breast 6
months later, the histological examination revealing a papilloma
and atypical dysplasia, and another patient had inflammatory
alterations at the histological examination, which could explain
a release of epithelial cells.

In group 2, eight patients (24%) where positive with a Ct
<37. All had an invasive ductal breast carcinoma, with high
grade (SBR II or III), with or without nodal involvement. In
group 3, 27 patients (45%) had a Ct <37 and, among them, 12
patients (52%) had a metastatic breast cancer. The increasing
frequency of positive detection with aggressivity of the disease
supports the specificity of the method. In addition, Cts in group
3 were lower than in group 2, and the number of positive
replicates was higher than that in group 2. In addition, some
patients had a positive detection in all replicates (15%), sug-

4 Unpublished data.

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P. de Cremoux, J. M. Extra, M. G. Denis, et al.


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