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 blood-borne cells in breast cancer, especially at the operable stage, may be investigated by following these patients.

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.

The regulation of MUC1 expression is not currently elucidated, but the function of MUC1 is evoked in cell adhesion and metastasis (9–12). We show that by combining immunomagnetic enrichment and RT-PCR, we can specifically detect with high sensitivity of one MUC1-expressing cell in 5-ml blood samples. We then analyzed blood samples from 122 patients with breast disease.

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)]

3 The abbreviations used are: RT-PCR, reverse transcription-PCR; Ct, cycle threshold.

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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 tumor. 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 chemotheraphy 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, chemotheraphy, 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 with 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 nucleotide sequences for MUC1. MUC1 forward and reverse primers in 5’ and 3’ orientations were GTCGCCCCTAGCAGTACCCG (exon 6) and GACGTGCCCTACAGTTGG (exon 7). The Taqman probe (AGCCCCCTATGAGAAGGTTTCTGCAGGTAATG, exons 6–7) carrying a 5’ FAM reporter label and a 3’ TAMRA quencher group was synthesized by PE Applied Biosystems (Warrington, United Kingdom).

Of the total reverse transcription volume of 20 μl, 5 μl were used for each PCR. The polymerase amplification was performed in a total volume of 50 μl containing 1× PCR buffer [5 mM MgCl₂; 10 mM Tris-HCl (pH, 8.3); 50 mM KCl; 5% glycerol; 200 μM dATP, dCTP, and dGTP; 400 μM dUTP; 200 nM of each primer and 400 nM probe; 1.25 units of AmpliTaqGold DNA polymerase (PE Applied Biosystems); and 0.5 unit UNG]. The thermal cycling conditions comprised 2 min at 50°C, 10 min at 95°C, and then 45 cycles at 95°C for 15 s and 60°C for 1 min.

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).

**RESULTS**

Detection of MUC1 Transcripts in Peripheral Blood Samples. Without immunomagnetic separation, peripheral blood of healthy donors lead to an amplification of illegitimate MUC1 transcripts with a Ct of around 33 (n = 5 samples). The immunomagnetic separation lead to the absence of amplification signal (Ct, 45). Microscopic observation after immunomagnetic

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Distribution of patients and samples</th>
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<tr>
<td></td>
<td>Pretreatment</td>
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<tr>
<td>Group 1</td>
<td>28</td>
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<td>Group 2</td>
<td>34</td>
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<td>Group 3</td>
<td>60</td>
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<td>All</td>
<td>122</td>
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<th>Table 2</th>
<th>Histological data of group 1 patients</th>
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<tr>
<td>Histology</td>
<td>No. of patients</td>
</tr>
<tr>
<td>Fibroadenoma</td>
<td>11</td>
</tr>
<tr>
<td>Macro cysts</td>
<td>2</td>
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<tr>
<td>Normal mammary gland</td>
<td>1</td>
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<tr>
<td>Duct ectasia</td>
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<tr>
<td>Epithelial hyperplasia</td>
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<tr>
<td>Papilloma</td>
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<tr>
<td>Phyllodes tumor</td>
<td>1</td>
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<tr>
<td>Fibrocystic mastopathy</td>
<td>5</td>
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A consistent detection of 5 pg of RNA was observed (Table 3 and Fig. 1), potentially detecting a single breast cancer cell (estimated 5–10 pg). These results were confirmed by the dilution of a known number of MCF7 cells in 5 ml of normal human blood samples. The spiked samples were processed as described in “Material and Methods,” including the immunomagnetic separation. The observed Cts were 33.73, 35.37, and 37.75 for 20, 2, and 1 cells, respectively, diluted in 5 ml of blood (results of four duplicate independent experiments). A single cell was consistently detected in these conditions.

These results allowed us to determine the Ct for one-cell detection to be 37. Above this threshold, samples were considered as negative.

**Detection of MUC1 Transcripts in Peripheral Blood of Patients with Benign Breast Disease (Group 1).** Group 1 comprised 28 patients with benign breast disease analyzed in blind. The mean age of these patients was 42 years (range, 24–55). Of these 28 patients, 3 (11%) were considered positive, with a Ct equal to 37, all three in only one of the four determinations, indicating of a very low level of MUC1 transcripts in the sample. Seven patients of group 1 were also analyzed 24 h after surgery; none of them had detectable MUC1 transcripts. One patient was positive (Ct, 37) before surgery and negative after surgery. Two patients had a fibroadenoma and one fibrocystic mastopathy. No clinical history of malignancy was found.

**Detection of MUC1 Transcripts in Peripheral Blood of 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 T1 and T2 breast cancer patients who underwent primary surgery with or without adjuvant chemotherapy or radiotherapy; group 3 (60 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 (χ² 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 (carcinoembryonic 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 mononuclear 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 ($\Delta Rn$). 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.
The histological diagnosis confirmed that there was no
MUC1 involvement. A significant correlation between the frequency of
advanced breast tumors (inflammatory breast cancer), patients
patients with operable breast tumors (group 2); and (b cancer patients were analyzed: (a) patients 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 fibroadenoma, 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%), suggesting that the level of MUC1 transcripts is higher than in group 2.

In conclusion, immunomagnetic separation of epithelial cells from blood of breast cancer patients, combined with real-time RT-PCR of MUC1 transcripts is a noninvasive, sensitive, and specific assay for the detection of circulating mammary cells. The malignancy of such cells, as well as the prognostic value of that assay, has to be established with other molecular probes and with the follow-up of the patients. In patients with advanced breast cancer, the assay will be evaluated as a surrogate end point for assessing the efficacy of chemotherapy.

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Unpublished data.


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