Detection of Epithelial Messenger RNA in the Plasma of Breast Cancer Patients Is Associated with Poor Prognosis

Tumor Characteristics

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ABSTRACT

**Purpose:** Free plasma RNA has been scarcely studied in patients with cancer. Here we examine the presence of RNA from epithelial tumors in plasma from a series of breast cancer patients and its correlation with tumor characteristics and circulating tumor cells.

**Experimental Design:** β-actin mRNA was analyzed to check the viability of plasma RNA in samples from 45 patients with breast cancer and 25 controls. Nested primers were used to detect the presence of cytokeratin 19 (CK19) and Mammaglobin in the same samples. Eleven clinicopathological parameters were studied and correlated with molecular parameters. Additionally, we looked for circulating tumor cells in 16 of these patients and in 10 of the controls.

**Results:** All samples showed detectable quantities of β-actin RNA. In controls, 3 cases (12%) were positive for Mammaglobin, and 5 (20%) were positive for CK19 RNA; of the 45 patients, 27 cases (60%) were positive for Mammaglobin, and 22 (49%) were positive for CK19. These differences were statistically significant (P = 0.001). Tumor size (P = 0.01) and proliferative index (P = 0.02) were associated with the presence of Mammaglobin, CK19, or both RNAs in plasma. Pathological stage (P = 0.06) was close to significance. Although a statistical relationship was not demonstrated, 9 of the 10 patients with circulating tumor cells showed epithelial mRNAs in plasma.

**Conclusions:** We conclude that epithelial tumor RNA is detectable in plasma from breast cancer patients and that this finding is associated with a probable poor prognosis and circulating tumor cells.

INTRODUCTION

In most developed and in many developing countries, breast adenocarcinoma is the most common malignancy in the female population (1). Although long-term survival from breast cancer has not varied since the 1980s (2), its prevalence rate has increased, partly as a result of the aging population, and qualitative changes in life style constitute risk factors (3). A potential strategy for improving the survival rate would be the development of techniques to detect metastatic or recurrent disease in preclinical or symptomatic phases.

In recent years, reports have identified free circulating tumor DNA in peripheral blood of tumor patients, including those of higher prevalence, such as lung cancer (4–6), breast carcinomas (7–9), and colon cancer (10–12). In breast cancer, a statistical relationship between poor prognosis and the presence of tumor DNA has been reported (7); although the implications of tumor plasma DNA for disease outcome are still being evaluated; a correlation with poor survival is expected, as has been found in pancreatic carcinomas (13) and esophageal adenocarcinomas (14).

In addition to DNA, RNA has also been found circulating in the plasma and sera of healthy subjects (15, 16), and increased levels have been observed in cancer patients (17–19). Tyrosinase mRNA has been detected in patients with metastatic malignant melanoma (20), and cell-free EBV-associated RNA has been detected in the plasma of patients with nasopharyngeal carcinoma (21). Recently, the presence of amplifiable telomerase RNA (telomerase RNA template and telomerase reverse transcriptase protein) has been confirmed in serum samples from breast cancer patients (22). None of these studies has compared the presence of mRNA tumor markers in plasma or serum with other clinicopathological characteristics of the tumors.

The present study had two aims: (a) to analyze the distribution of 11 clinicopathological parameters in a larger series of breast cancer patients with respect to the presence or absence of plasma tumor RNA; and (b) to examine the relationship between the presence of circulating tumor cells and the detection of specific tumor mRNA in plasma.

MATERIALS AND METHODS

**Plasma Samples and RNA Extraction.** Informed consent was obtained from all participants, and the study was approved by the research Ethics Board of our hospital. Blood samples from 45 patients with breast cancer were collected at time of surgery. Plasma was prepared by centrifugation of peripheral blood at 1000 × g for 20 min and then divided into...
 aliquots. These were then snap frozen at -80°C until processing. Blood samples of 25 healthy controls were also obtained.

RNA was extracted from 3 ml of plasma using a commercial kit, according to the manufacturer’s protocol Rneasy Mini Kit (Qiagen, Inc., Hilden, Germany), with the following modifications: 3 ml of plasma sample were mixed with 600 μl of buffer RLT and 6 μl of β-Mercaptoethanol; after that, 3.5 ml of ethanol 70% were added and cooled to 4°C for 30 min. A final centrifugation was carried out at 1500 × g for 5 min, and the supernatant was collected. One column was used repeatedly until the whole sample had been processed. The concentration of RNA was then measured by spectrophotometry.

**Clinicopathological Parameters Analyzed.** Date of birth, histological type, tumor size, lymph node metastases, peritumoral vessel involvement, pathological stage, histological grade, oestrogen and progesterone receptor status, proliferative index, and TP53 IHC status were all analyzed.

Pathological stage was assessed using the Tumor-Node-Metastases classification. All tumors were graded according to the Bloom-Richardson grading system. The steroid receptor content (oestrogen and progesterone) was determined by an IHC³ procedure, the results of which were considered positive when ≥25% of the cells stained positively. The proliferative index of the tumors was demonstrated by Ki-67 antigen (Immunotech, Westbrook, ME) in IHC analysis; the Ki-67 index was considered high when ≥20% of the cells stained positively. Mutational status of TP53 was analyzed by immunohistochemistry with the cl 1801 mouse monoclonal antibody (Oncogene Science, Manhasset, NY). Cases exhibiting definitive nuclear (or nuclear and cytoplasmic) staining in >10% of the epithelial cells were considered positive for p53.

The immunostaining procedures were performed, in every case, on paraffin-embedded material using retrieval techniques. Normal breast tissues were used as internal primitive controls in hormonal receptor studies. Ki-67- and p53-positive cases were considered positive for p53.

**Circulating Tumor Cells Analysis.** The study of micro-metastases in blood was performed in three steps. First, 10 ml of blood were taken from 16 patients and 10 controls; MNCs were obtained from these samples using a ficoll solution, (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). Then, the MNC fraction was enriched by a positive isolation method (Dyna-beads epithelial enrichment protocol; Dynal AS, Oslo, Norway) in epithelial circulating tumor cells (23, 24). As a result, the circulating tumor cells were enriched by more than three logs from MNC. Finally, RNA was extracted using the commercial Rneasy Mini Kit. Tumor cell dilution experiments showed that magnetic beads enrichment protocol links with RT-PCR displayed a sensitivity of 1 cell/ml of blood (data not shown).

**RT-PCR and Primers Condition.** RNA (between 5–15 ng) from plasma and cells was reverse transcribed and amplified using the GeneAmp gold RNA PCR Core Kit for RT-PCR (PE Biosystems, Foster City, CA) and then amplified by PCR in a one step, according to the manufacturer’s protocol, with variable annealing temperatures, depending on the pair of primers. The final reaction volume was 10 μl. Amplification was performed in a thermal cycler (Perkin-Elmer/Cetus, Foster City, CA).

Three human genes were amplified with this protocol: β-actin, CK19, and Mammmaglobin. A second nested PCR was performed because the signal obtained from plasma RNA was weak. β-actin was used to verify the presence and integrity of serum RNA because storage can modify the results (20).

**β-actin Primers.** Bf (outer, sense) 5’CCAACCCGGAG-AAGATGACC3’; Br (outer, antisense) 5’TGGCCAAATGGGTAT-GACCTGG3’. Bnf (nested, sense) 5’GATCATGTTTGAGAC-CTTCC3’; Bnr (nested, antisense) 5’GTCAOGCAGCTGTAQ3’. PCR conditions for outer primers were: 95°C for 9 min followed by 40 cycles of 95°C for 20 s, 64°C for 15 s, 72°C for 30 s, and finally, 72°C for 11 min. Nested PCR conditions were: 95°C for 9 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and finally, 72°C for 11 min.

**CK19 Primers.** CKf (outer, sense) 5’GTGAGGATT-ATTCGCCCTCC3’; CKr (outer, antisense) 5’TGGCAATCTCCTC- TGCTCCAGC3’. CKnf (nested, sense) 5’ATGCGCAGACAG- AACCAGAA3’; CKnr (nested, antisense) 5’CCATGAGCCGCTGTA-CTTCC3’. PCR conditions for outer primers were: 95°C for 9 min followed by 43 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min, and finally, 72°C for 11 min. Nested PCR conditions were: 95°C for 9 min followed by 40 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 30 s, and finally, 72°C for 11 min.

A set of primers designed by Dynal AS was used to maximize mismatch between the CK19 gene and the two processed pseudogenes. A nested PCR of genomic DNA dilutions showed that no pseudogene amplification occurred.

**Mammaglobin Primers.** Manf (outer, sense) 5’CAGCGGCTTCCTTTGATCG3’; Manr (outer, antisense) 5’TAG- CAGGTTTTACAATTTGTC3’. Mannf (nested, sense) 5’AGCG- ACTGCTACGCAGGCTCT3’; Mammr (nested, antisense) 5’ATAAGAAGAGAAGGTTGGG3’. PCR conditions for outer primers were similar to those of CK19, excepting annealing temperature that was of 55°C.

All nested PCRs were amplified using 1.2 μl from the first PCR amplification in a final volume of 10 μl in the presence of 0.3 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, NJ), 1 μl of 10 × PCR buffer, 200 μM deoxynucleotide triphosphate, 0.6 μM of each primer, and different concentrations of MgCl₂, depending on the gene. All primers were designed expanding intron-exon junction.

Amplification conditions were designed to minimize the possibility of positive results in controls. Amplifications included a positive control (Mammmaglobin or CK19) consisting of mRNA from breast tumor tissue and a negative control lacking cDNA. Duplicate experiments were concordant in >94% of samples. Discordant cases were repeated again to clarify the result. Finally, seven samples were sequenced to check if the amplimers were correct and all of them confirmed the expected sequence (ABI PRISM 377; Perkin-Elmer/Cetus).
Detection. The final 10 μl of the PCR product was mixed with a 3-μl volume of loading buffer (total volume, 13 μl), 0.02% xylene cyanol, and 0.02% bromphenol blue. Electrophoresis was run on nondenaturing 8–12% polyacrylamide gels for 2 h at 500 V. After gel electrophoresis, the band intensity was detected by a nonradioisotopic technique using a commercially available silver staining method (25).

Statistical Analysis. The variables analyzed were compared using the $\chi^2$ test with Yates correction or Fisher’s exact test when any of the expected frequencies was <5. Two-tailed $P$ values of <0.05 were considered significant. Statistical analyses were performed using the EPI-INFO package, version 6.04.

RESULTS

The Isolated Amount of RNA from Plasma Ranged between 50–200 ng/ml. When the viability of the RNA samples was analyzed via the amplification of $\beta$-actin, it was found that all plasma samples from controls and patients presented detectable quantities of $\beta$-actin mRNA showing an acceptable integrity to amplification (Fig. 1).

Subsequently, the presence of Mammaglobin and CK19 mRNA in plasma samples was assessed. Three (12%) of the 25 control samples were positive for Mammaglobin mRNA, whereas 27 of 45 (60%) samples from tumor patients were positive. CK19 mRNA was found in 5 of 25 controls (20%) and in 22 of 45 (49%) samples from patients (Fig. 1).

When the distribution of these epithelial mRNA markers in the plasma of controls and patients was analyzed, 8 (32%) of the controls were positive for at least one marker, and the remaining 17 (68%) samples were negative for both markers. On the other hand, 33 (73%) of patients were found to be positive for at least one marker, whereas 12 (27%) were negative for both epithelial markers. The difference in distribution between the groups was statistically significant, $P = 0.001$ (Table 1).

Upon comparing clinicopathological parameters with molecular results, five of them were found to be consistently related to the presence of epithelial tumor mRNA (one or both of the markers used, CK19, Mammaglobin). Two of the tumor characteristics revealed a significant difference: tumor size ($P = 0.01$) and proliferative index ($P = 0.02$); a third parameter, pathological stage ($P = 0.06$), was close to significance (Table 2). None of the remaining clinicopathological parameters revealed statistically significant differences.

On the basis of these results, we also looked for circulating tumor cells in 16 of these patients and 10 controls. None of the 10 controls tested had epithelial mRNA in MNCs. On the other hand, 10 (62.5%) of the MNCs from the 16 patients analyzed were positive for the markers studied, 9 with Mammaglobin and 1 with both markers. However, when the results obtained in plasma and MNCs in patients were compared, no significant relationship was revealed. 9 of the 10 patients with circulating tumor cells having epithelial mRNAs in plasma ($P = 0.1$; Table 3).

DISCUSSION

Improvements in molecular and genetic analysis have enabled the identification of tumor-derived nucleic acids circulating in the plasma of cancer patients (4–22). Although the mechanisms by which these nucleic acids are released into plasma remain unknown, there are several hypotheses about their origin (lysis, tumor necrosis, apoptosis, and active shedding; Refs. 26–29).

Kopreski et al. (20) detected tyrosinase mRNA sequences in the serum of several patients with metastatic malignant melanoma, whereas no tyrosinase mRNA was detected in the control group. Lo et al. (21) reported the presence of EBV-associated RNA in plasma of patients with nasopharyngeal

Table 1 Distribution of mRNA epithelial markers (CK19 and Mammaglobin) detection in controls and patients

<table>
<thead>
<tr>
<th>Markers</th>
<th>Controls</th>
<th>Patients</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>17</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>33</td>
<td>0.001</td>
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</table>

Table 2 Statistical distribution of tumor characteristics with respect to the presence or absence of tumor mRNA in plasma, characterized by the positivity or negativity of markers used

<table>
<thead>
<tr>
<th></th>
<th>With mRNA</th>
<th>Without mRNA</th>
<th>P</th>
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<tbody>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤3</td>
<td>12</td>
<td>10</td>
<td>0.01</td>
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<td>&gt;3</td>
<td>21</td>
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<td>+</td>
<td>16</td>
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<tr>
<td>III</td>
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<td>1</td>
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carcinomas and in a small subset of controls, and more recently, Chen et al. (22) showed that it was possible to amplify telomerase RNA template and telomerase reverse transcriptase protein mRNA in the serum of breast cancer patients.

Similarly, our work shows that the presence of CK19 and Mammaglobin mRNA in plasma is closely related to patients with epithelial-tumor-derived, this being a significant association ($P = 0.001$). Although some of these markers can be found in plasma of the control group, this could be attributable to conditions such as the minimal illegitimate levels of CK19 expression in mononuclear blood cells (30). Furthermore, none of the controls revealed the concomitant presence of these two markers in plasma, whereas 16 (35.5%) of the breast cancer patients did.

The present study is the first description of breast cancer patients with circulating tumor mRNA to show a significant relationship to poor prognostic features, increased size, proliferative index, and histological stage. Regarding our patients with circulating tumor cells, 90% had positive plasma samples for tumor mRNA; a similar relationship was found in colon cancer (data not shown). In addition, 3 patients with no signs of circulating tumor cells had a positive epithelial marker in plasma. The relationship between tumor cells in blood and tumor nucleic acids in plasma remains unclear. Extracellular DNA and RNA from circulating cells may have easier access to the blood than nucleic acids from primary tumor cells. Alternatively, the shedding capacity of these potentially metastatic circulating cells may have been much higher, as suggested by differences in the fluidization of plasma membrane between metastatic and nonmetastatic neoplastic cells (31).

One approach to understanding plasma tumor nucleic acids is the identification of patients with good prognosis but poor outcome, and it has been demonstrated that patients with early stage tumors and circulating tumor DNA show lower survival rates (32). The recurrence rate in stages I and II breast cancer is between 10–20% and 40–65%, respectively (32). In our series, a high proportion of patients with early stages had plasma tumor mRNA, which may enable us to study the influence of this molecular parameter on patient survival and recurrence rate. To date, no studies have been reported analyzing the correlation between plasma mRNA and patient outcomes.

In conclusion, as with specific DNA tumor alteration, the detection of tumor-related mRNA in patients with breast cancer is a reliable fact. Moreover, a positive correlation with poor pathological parameters, consistent with more aggressive tumors, has been observed in patients with tumor mRNA present in plasma. In addition, a clear relationship was revealed between plasma tumor RNA and the presence of circulating tumor cells.

Additional studies with other malignancies are needed to test the potential of this new plasma molecular marker.

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