Identification of Superior Markers for Polymerase Chain Reaction Detection of Breast Cancer Metastases in Sentinel Lymph Nodes

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Abstract

Sentinel lymph node biopsy (SLNB) is being evaluated in breast cancer patients to improve detection of metastases and to guide therapy with minimal morbidity. The use of reverse transcription-PCR analysis to increase detection of tumor cells in SLN of breast cancer patients is hampered by the lack of specific markers. In this study, seven markers were evaluated by reverse transcription-PCR for expression in human breast adenocarcinoma lines (BrCa) and in normal nodes from non-cancer patients. Two markers yielded exceptional results; mammaglobin and carcinoembryonic antigen transcripts were detected in 100 and 71% BrCAs, respectively, and were absent from all normal lymph nodes. These markers will be used as components of a multimarker panel to evaluate sentinel nodes in an on-going, multicenter clinical trial.

Introduction

The single most important prognostic indicator in cancer patients with solid tumors is the detection of disease in lymph nodes. SLNB is a minimally invasive surgical method of mapping the lymphatic route of tumor cells to the one or two lymph nodes (the sentinel nodes) that primarily drain the tumor and thus are most likely to harbor metastatic disease. This procedure was pioneered by Giuliano et al. (1) in melanoma patients using peritumor injection of a blue vital dye to locate the sentinel node. The additional injection of technetium sulfur colloid (and the intraoperative use of a handheld gamma probe) presently provides the most sophisticated technique for SLN localization. SLNB is now accepted as standard of care for melanoma patients, and complete lymph node dissection is reserved only for those patients with a histologically positive SLN. SLNB involves only minimal surgery under local anesthesia and avoids the morbidity associated with complete lymph node dissection, including pain, increased hospital stay, and acute and chronic lymphedema.

We are investigating the extension of this technique to breast cancer patients. Current treatment for breast cancer includes the complete removal of 10–30 axillary lymph nodes. After surgery, one section of each lymph node is typically examined by pathologists for the presence of disease. Sixty % of these patients are found to have disease-free lymph nodes upon routine H&E analysis and thus have been subjected to unnecessary surgical and medical risks (2). In addition, this pathological analysis results in a high false-negative rate; one-third of women with pathologically negative lymph nodes develop recurrent disease (2).

SLNB allows a more directed and comprehensive search for metastases in one or two SLN, in contrast to the present limited analysis of the multiple lymph nodes from a complete dissection. The identification of the SLN allows the entire tissue to be serially sectioned and evaluated for the presence of metastatic disease, both by routine histology and by immunohistochemistry. SLNB also enables the application of the more sensitive PCR technique, which typically detects one tumor cell in 10^6 normal cells.

To date there have been few studies that specifically address PCR analysis of SLN in breast cancer patients. Most studies of breast cancer patients have analyzed undefined axillary lymph nodes or have analyzed inappropriate "normal" control tissue. An ideal breast marker (present on all tumor cells from all patients and always absent from normal lymph nodes) has not been identified, hampering PCR applications. Rather than prematurely perform PCR analyses on breast cancer patient samples, we have concentrated on defining the optimal markers. Because it is unlikely that one single marker will be present in malignant SLN of all patients with breast cancer, we hypothesized that the use of a multimarker panel would increase the likelihood of cancer detection across the population. The objective of this study was to develop a panel of markers for RT-PCR detection of breast cancer micrometastases in SLNs. Marker candidates included tumor- and tissue-specific antigens, as well as proteins associated with angiogenesis and metastasis. A group of human breast cancer cell lines and lymph nodes from normal, non-cancer patients were screened for expression of these potential markers.

Materials and Methods

Cell Lines and Tissue Samples. The human breast cancer cell lines BT-20, T-47D, MCF-7, MDA-MB-231, MDA-MB-436, MDA-MB-468, and ZR-75-1 were obtained from the American Type Culture Collection and cultured as described by American Type Culture Collection. Control lymph nodes were obtained from non-cancer patients as by-products of several surgical procedures. Patient tissue samples were obtained as part of an ongoing multicenter clinical trial, following an established protocol that has received Human Investigation Review Board approval from all investigative sites. Informed consent was obtained from each subject. Banked specimens from all cancer patients (SLN and non-SLN, tumor) are stored at −70°C until RNA extraction.

PCR Facility. The PCR facility is segregated into physically and functionally discrete laboratories for RNA/DNA isolation, PCR reaction set-up, DNA amplification, and postamplification analysis. The reaction set-up room contains a TemplateTamer workstation (Oncor Corporation), which allows reactions to be set up in an aerosol-free environment and enables UV irradiation of the work area, dedicated pipettes, filter tips, and reaction tubes.

RNA Isolation. Cultured cells were washed with PBS and frozen at −70°C as pellets. Extraction was performed according to the manufacturer's instructions using 1 ml of TRizol (Life Technologies, Inc.).10^6 cells. Frozen tissue specimens were cooled in liquid nitrogen and pulverized with a mallet before homogenizing in TRizol (1 ml/50 mg of tissue) using a Polytron generator (Brinkman). The homogenization probe is thoroughly decontaminated between samples by a series of detergent washes and base hydrolysis steps. Rigorous
precautions were taken to prevent RNA degradation by RNase, including the use of gloves, baked instruments, and diethylpyrocarbonate treatment of liquids to inactivate the RNase. RNA integrity was verified by the predominant intact presence of 18S and 28S RNA bands on a denaturing formaldehyde gel.

cDNA Synthesis. RNA (1–5 μg) was incubated with 0.5 μg of oligo(dT)~18 primer at 65°C for 7 min and chilled on ice before addition to a 20-μl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 0.5 mM dNTP, 5 units of cloned plasmid RNase inhibitor, and 200 units of Maloney murine leukemia virus reverse transcriptase. Samples were incubated for 1 h at 37°C, followed by heating at 94°C for 5 min. Lymph node cDNA samples were diluted 1:5, and BrCa cDNA samples were diluted 1:2.5 with water prior to freezing.

PCR. PCR was performed using published primer sequences as described for K19 (3), MUC-1 (4), maspin (5), VEGF (6), TGF-β (Clontech Corp., Palo Alto, CA), CEA (7), and mammaglobin (8). Life Technologies reaction buffer was used for all samples (final concentration, 20 mM Tris-HCl, 50 mM KCl). Other reaction components were 2.5 mM MgCl₂, 2 mM dNTP, 0.4 mM each primer, and 2 units of Taq polymerase (Life Technologies, Inc.) with the following exceptions: VEGF reactions contained 1.25 mM MgCl₂, maspin reactions contained 1 unit of Taq and 1.25 mM dNTPs, and mammaglobin reactions used 1 unit of Taq polymerase. Annealing temperatures/times were as described, except as follows: 65°C for 45 s for K19 and MUC-1; and 55°C for 1 min for maspin, VEGF, and mammaglobin. Extension temperatures/times were as described, except was 72°C for 2 min for K19, MUC-1, and mammaglobin. An updated mammaglobin primer set (sense, 5'-AGCACTGCTACGCAG-3'; antisense, 5'-ATAAGAAAGAGAAGGTGTGG-3') was obtained midway through the study and used at 0.64 mM in PCR reactions with 1.5 mM MgCl₂ and 58°C for 1-min annealing times.

Four μl cDNA was used in a 50-μl reaction, and amplification was performed in a Perkin-Elmer 480 thermal cycler, which was heated to 94°C before loading samples. For mammaglobin and CEA, a 2-min initial preincubation at 94°C preceded the normal cycling pattern. Forty cycles of amplification were performed for all primers except for nested reactions for CEA (20 + 20 cycles) and maspin (35 ± 35 cycles). PCR-amplified products (10 μl) were analyzed by submarine electrophoresis on 1.8% 3:1 NuSieve agarose gels and visualized by ethidium bromide staining.

PCR Reaction Controls and Interpretation Criteria. Primers were selected to span introns, so that any contaminating genomic DNA would not be amplified. It is unknown whether the maspin primers span an intron, because only cDNA sequence information was available for maspin. In the absence of pseudogenes, amplified genomic DNA would be evident as a much larger PCR product (generally 2-3 kb), compared with the specific products amplified from cDNA templates (0.131–0.460 kb). Any contaminating genomic DNA in RNA preparations would also be evident from the RNA formaldehyde gel electrophoresis analysis described above, because genomic DNA does not move from the electrophoresis origin. As an additional test for genomic DNA contamination, PCR reactions were conducted using RNA preparations in place of cDNA; any amplified PCR product would thus be derived from contaminating genomic DNA. On the rare occasion when genomic DNA contamination was evident, the RNA samples were treated with DNase (Life Technologies, Inc., 3 units/μg RNA) and reevaluated prior to cDNA synthesis.

As a positive PCR control, cDNA preparations were amplified in parallel reactions with β-actin primers (9) concurrent with each experimental primer set to control for the integrity of the amplification reaction components and to determine whether the cDNA preparation contained amplifiable DNA template. PCR results from the master panel were used only if the β-actin reaction resulted in amplified PCR products of the appropriate size.

PCR reaction set-ups contained an "open tube" control without a cDNA template, which was included in every PCR amplification set as a negative control to insure that samples were not contaminated with exogenous DNA. PCR results from the master panel were used only if the "open tube" reaction resulted in no detectable amplified PCR products upon gel electrophoresis. As a positive control for the experimental marker primers, PCR analyses of lymph node samples included at least one human breast cancer cell line that was positive for the given marker.

Final acceptance of PCR data required consistent results from at least three separate PCR reactions using at least two different cDNA preparations.

PCR Detection Sensitivity. Serial dilutions of ZR-75-1 BrCa cells (5 × 10⁵–5 × 10⁸ cells) were mixed with lymphocytes (5 × 10⁹) obtained from normal lymph nodes of non-cancer patients. RNA extraction and PCR analysis was performed as described above.

Results and Discussion

The markers reported in this study were selected based largely on their previously reported specificity for breast cancer, as determined by immunohistochemistry or PCR. The rationale is briefly summarized below.

Keratins are components of intermediate filaments in epithelial cells and common targets in immunological staining of cancer specimens. The cytookeratin K19 is most often cited as a superior marker for breast neoplasms and has been reported as a promising sensitive marker for the detection of breast cancer micrometastases in axillary lymph nodes (3, 10, 11). MUC-1, an epithelial membrane-bound mucin critical in cell adhesion, is reported to be overexpressed in breast cancers and absent from normal lymph nodes (12). A RT-PCR study with MUC-1 primers detected micrometastases in 15% histologically negative lymph nodes (4), although its sensitivity was lower than K19 in a direct comparison (13).

Serine protease inhibitors (serpins) have been implicated in the progression of breast cancer. Maspin, a mammary serpin, was an attractive candidate because maspin transcripts were detected in primary and metastatic breast tumor samples and absent from peripheral blood samples of normal donors (5). Two growth factors implicated in tumor progression were also evaluated. VEGF is elevated in a variety of cancers and is thought to play a pivotal role in angiogenesis of new tumors. TGF-β is a cytokine that has been implicated in the regulation of breast cancer proliferation and in the support of tumor stromal growth. A study of TGF-β expression in breast cancer patients found identical results with immunohistochemistry and RT-PCR, endorsing PCR as a desirable procedure for analysis of small samples (14).

CEA is a notable tumor marker for gastric and colorectal cancer that is also expressed by the majority of breast tumors. There are reports of CEA as an RT-PCR marker to detect carcinoma cells in lymph nodes (7, 15), bone marrow (16), and peripheral blood (15) in breast cancer patients. Most promising was the report that the detection of positive axillary lymph node increased from 26% via histological analysis to 66% by RT-PCR analysis for CEA (7).

Mammaglobin is a recently described tissue-specific marker, expressed only in adult mammary tissue and in 80–95% breast primary tumors, where it is frequently overexpressed (8). Mammaglobin mRNA has been detected in over 60% of axillary lymph nodes of patients with metastatic breast cancer and in peripheral blood stem cells from 60% of breast cancer patients but not in normal lymph nodes from non-cancer patients.

Each of these potential markers was screened for expression in seven human breast cancer cell lines of varying immunogenecity and metastatic potential and in lymph nodes from normal, non-cancer patients. Human breast cancer cell lines were selected based on their usage in cancer research and their diverse origins with respect to the type of cancer and the patient source. MDA-MB-231, MDA-MB-436, MDA-MB-468, and MCF7 were isolated from pleural effusions of breast adenocarcinomas. T-47D and ZR-75-1 were from effusions of infiltrating ductal carcinomas. BT-20 was isolated from a primary infiltrating ductal carcinoma.

The results of the RT-PCR analysis are summarized in Table 1. Although K19, MUC-1, maspin, VEGF, and TGF-β were expressed in all of the human breast cancer cell lines tested, they were also expressed in 30–100% of normal lymph nodes from non-cancer patients. It is apparent that these markers lack the necessary specificity

5 T. Fleming, personal communication.
PCR MARKERS FOR BREAST CANCER AND SENTINEL NODES

Table 1 Summary of RT-PCR analysis of human breast carcinoma cell lines and normal lymph node tissue for expression of tumor markers

<table>
<thead>
<tr>
<th>Tumor markers</th>
<th>K-19</th>
<th>MUC-1</th>
<th>Maspin</th>
<th>VEGF</th>
<th>TGF-β</th>
<th>CEA</th>
<th>Mammaglobin</th>
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<tr>
<td>BrCa cell lines</td>
<td></td>
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</tr>
<tr>
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<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
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<td>Positive in normal lymph nodes</td>
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<td>0/20</td>
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</table>

* +, indicates specific PCR product detected from at least two cDNA preparations and three PCR reactions.

Fig. 1. Lack of detectable CEA and mammaglobin expression in normal lymph nodes from non-cancer patients by RT-PCR analysis. BrCa line ZR-75-1 served as a positive control for marker primers. Expression of ß-actin served as a positive control for cDNA preparations and PCR reaction conditions. PCR products were electrophoresed on 1.8% agarose gels and visualized by ethidium bromide staining.

Fig. 2. CEA and mammaglobin expression in human breast carcinoma cell lines detected by RT-PCR analysis. Expression of ß-actin served as a positive control for cDNA preparations and PCR reaction conditions. PCR products were electrophoresed on 1.8% agarose gels and visualized by ethidium bromide staining.

We have initiated a multicenter clinical trial with breast cancer patients to determine whether SLNB analysis could predict the presence of disease in the remaining axillary lymph nodes. The trial has accrued 140 patients to date with 13 active investigative sites. The protocol requires SLNB followed by full node dissection in all patients. We have recently begun to analyze specimens from this trial using mammaglobin and CEA primers with promising preliminary results. One example of RT-PCR analysis of a breast cancer patient's specimens is shown in Fig. 3. In this patient, both the tumor and the histologically positive SLN were positive for mammaglobin expression, and the histologically negative, non-SLN was negative for mammaglobin expression. Clearly, immunohistochemistry and PCR results from patient specimens must be compared for concordance in this trial as analytical data accrues. Our preliminary results do indicate that PCR analysis with these markers confirms immunohistochemistry-positive SLN results and that, in some cases, PCR analysis detects marker expression in immunohistochemistry-negative SLN. Long term follow-up studies will be essential to determine whether those patients with PCR-positive, histologically negative sentinel nodes are at greater risk for disease recurrence and to determine whether PCR-negative sentinel nodes are associated with a good prognosis. Promising results were reported recently in a 2-year follow-up of cancer patients with gastrointestinal or breast carcinomas who had undergone patient samples. This is the first report of the use of mammaglobin as a marker for the detection of sentinel node metastases (20).

for breast cancer to provide accurate analysis of clinical specimens. Although K19 has been used as a marker in ongoing breast SLNB trials, we detected K19 mRNA in 50% of normal lymph nodes. Even when the PCR assay sensitivity was decreased by reducing the number of amplification cycles from 40 to 30, a K19 RT-PCR product was still detected. Our results with K19 are consistent with recent data from other groups that also detected K19 in an unacceptable percentage of normal lymph nodes and normal peripheral blood lymphocytes (17-19).

In marked contrast to these markers, neither mammaglobin nor CEA mRNA was detected in any normal lymph nodes (see Fig. 1 for a representative analysis). Mammaglobin transcripts were detected in 100% of human breast cancer cell lines tested, although the level of expression varied greatly (see Fig. 2 for a representative analysis). CEA transcripts were detected in the majority (71%) of human breast cancer cell lines tested. The PCR sensitivity detection limit was found to be one tumor cell/10⁶ normal cells using mammaglobin primers and CEA primers (7). These exceptional results with mammaglobin and CEA render them superior markers for the testing of breast cancer

6 K. M. Verbanac, C. J. Min, and L. Tafra, unpublished data.
RT-PCR analysis of axillary lymph nodes for CEA (15). Although these investigators did not specifically isolate or identify the sentinel node, they found that PCR detection of CEA expression in axillary nodes was strongly associated with recurrence, even in historically negative specimens.

In conclusion, we have identified two outstanding candidates for multimarker RT-PCR analysis of sentinel lymph nodes in breast cancer patients. Our data indicate that the use of PCR analysis in clinical trials of SLNB in breast cancer patients should include CEA and mammaglobin as detection markers. We have demonstrated that certain markers presently in use will likely lead to an unacceptable false-positive rate. Future work needs to be done to identify other markers with the goal of obtaining a panel that can predict the prognosis of cancer patients with an accuracy and sensitivity not possible previously. It is hoped that SLNB, in conjunction with RT-PCR analysis using suitable markers, will lead to more accurate prognosis and to the selection of a surgical and medical treatment better tailored to each breast cancer patient.

Acknowledgments

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References


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