Detection of Cancer Cells in Peripheral Blood of Breast Cancer Patients Using Reverse Transcription-Polymerase Chain Reaction for Epidermal Growth Factor Receptor

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ABSTRACT
The epidermal growth factor receptor (EGFR) has been reported to be expressed in high levels in primary breast cancer by immunohistochemistry. In the present study, a reverse transcription (RT)-PCR assay using EGFR primers was developed and evaluated for the detection of circulating micrometastases in the blood of breast cancer patients. Total RNA was extracted from breast cancer cell lines and from the blood of 23 control individuals and 37 breast cancer patients. After reverse transcription, outer and nested primers for EGFR were used for cDNA amplification. RNA integrity was confirmed with parallel RT-PCR amplification using β2-microglobulin primers. PCR products were electrophoresed on agarose gels containing ethidium bromide and visualized by UV photography. Southern blotting was used to confirm EGFR specificity. The nested EGFR RT-PCR assay was capable of detecting a lower limit of 100 fg of total RNA from the A431 cell line. EGFR RNA was identified from the blood of 4 of 18 (22%) metastatic breast cancer patients, 0 of 6 locally recurrent breast cancer patients, 0 of 13 adjuvant breast cancer patients, and 0 of 23 controls (P = 0.03, metastatic versus control). The 18 metastatic breast cancer patients all had progressive disease at the time of blood sampling. The identity of the four EGFR-positive bands was confirmed by Southern blotting. The presence of RT-PCR positivity for EGFR was not a treatment-related phenomenon, because three of the four EGFR-positive patients were not receiving treatment at the time of blood collection. RT-PCR for EGFR is a sensitive and specific method for the detection of circulating micrometastases in a proportion of patients with metastatic breast cancer.

INTRODUCTION
Metastasis of cells from the primary tumor is the hallmark event in cancer progression. Animal experiments with solid tumors suggest that implantation of circulating tumor cells is an inefficient process, with only 0.01% of cells forming a metastatic deposit (1). Until recently, detection of these circulating cancer cells has been problematic because of their small numbers in comparison to peripheral blood cells, the limited survival of circulating cells (2), and the insensitivity of techniques available previously. The advent of PCR has led to rapid advances in the detection of cancer cells in the peripheral blood in lymphoma (3), neuroblastoma (4), melanoma (5), and prostate cancer (6). These advances have led to the suggestion that this technology may have a major impact on the treatment of many cancers (7).

In breast cancer, the hematogenous spread of metastatic cancer cells greatly influences the outcome of disease for most patients. The presence of micrometastatic breast cancer cells in the bone marrow of patients with primary breast cancer has been reported to confer a worse prognosis (8, 9). Since the original report describing the use of PCR for the detection of occult cancer cells in peripheral blood and bone marrow of breast cancer patients (10), several studies have confirmed the presence of micrometastatic tumor cells (11–15). Most of these studies were conducted using cytokeratin 19 primers for RT-PCR detection of the micrometastatic breast cancer cells (10–13). However, recent reports have concluded that RT-PCR of cytokeratins lacks the required specificity to be useful clinically, because a substantial number of false positives were seen in patients without cancer (13, 16–18). In addition to the problem of illegitimate transcription, cytokeratin 19 has a known processed pseudogene that further potentiates the possibility of false-positive RT-PCR results (19). These reports have led to the conclusion that caution should be used when interpreting RT-PCR results using cytokeratins (20).

The EGFR is expressed in glioblastoma and in a wide variety of cancers of epithelial origin including head and neck, esophageal, gastric, colon, lung, ovarian, prostate, bladder, and breast cancer (21). In breast cancer, the EGFR protein was reported to be overexpressed in 45% of patients in a review of 40 different studies with a combined total of over 5000 breast tumors (22). The majority of these studies used ligand-binding assays or immunohistochemistry (22). However, using RT-PCR, the EGFR mRNA was reported to be present in 88 of 94 (93%) primary breast cancers (23). Because the majority of...
primary breast cancers have detectable EGFR mRNA, we have developed and evaluated an RT-PCR assay using EGFR for the detection of circulating breast cancer cells.

PATIENTS AND METHODS

Patients. A total of 37 patients with breast cancer were included in this study. At the time of blood drawing, 13 patients were receiving adjuvant therapy after removal of the primary tumor, 6 patients had local recurrence of breast cancer, and 18 patients had metastatic disease. The metastatic breast cancer patients all had progressive disease; 9 patients were receiving treatment, and 9 patients were untreated at the time of blood collection. All patients gave written informed consent for blood drawing in a protocol approved by the Institutional Review Board of the Pennsylvania State University College of Medicine. A negative control group consisted of 23 healthy control individuals without cancer.

Cell Lines. The following human cell lines were from the American Type Culture Collection (Rockville, MD): A431 epidermoid carcinoma, SKBR3, MCF-7 and HS578T breast cancer, BxPC-3 pancreatic cancer, WiDr colon cancer, and SKOV3 ovarian cancer. BxPC-3 cells were grown in RPMI 1640, and WiDr cells were cultured in MEM; both media were supplemented with 10% FCS, 2 mM l-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin. All other cell lines were cultured as instructed by the supplier. Cells were grown at 37°C in humidified air containing 5% CO₂.

Blood Collection and RNA Extraction. Peripheral blood was drawn by forearm venipuncture using a 21-gauge butterfly needle infusion set. An initial serum Vacutainer tube was drawn to clear the line of any detached epithelial cells, followed by 20 ml of blood collected in EDTA Vacutainer tubes (Becton Dickinson, Rutherford, NJ). Within 2 h of collection, the EDTA-anticoagulated blood was lysed by the addition of RBC lysis buffer (0.155 M NaCl, 0.01 M KHCO₃, and 0.1 mM EDTA, pH 7.4) at a volume ratio of 3:1 lysis buffer: blood. The samples were incubated in an ice-water bath with occasional mixing for 10 min at 4°C. After RBC lysis, the remaining nucleated cells were collected by centrifugation at 1000 × g for 10 min at 4°C. The supernatant was aspirated, and the cell pellet was resuspended in 2 ml of TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD) per lysed pellet from 3 ml of blood. This solution was homogenized by vortexing and then frozen at −70°C. For cultured cell lines, 2 ml of TRIzol was added directly to the culture flask, the cell solution was homogenized by vortexing, and the solution was frozen at −70°C. Multiple frozen samples were then thawed, and total RNA extraction was performed as recommended by the TRIzol manufacturer. The total RNA pellet was resuspended in TE buffer (0.01 M Tris, 0.001 M EDTA, pH 8.0) and quantitated by spectrophotometry at 260 nm.

RT-PCR. Nucleotide primers were designed to span EGFR introns 1 to 7 and were derived from published sequence information (Fig. 1; Refs. 24 and 25). RT-PCR was performed using the GeneAmp RNA PCR kit (Perkin-Elmer, Foster City, CA) according to the manufacturer’s protocol. Total RNA (0.5–1.0 μg) was subjected to reverse transcription using 2.5 μM random hexamer priming, 5 mM MgCl₂, 1 mM each of deoxynucleotide triphosphates, 1 × PCR buffer II (50 mM KCl, 10 mM Tris-HCl, pH 8.3), 1 unit/μl RNase inhibitor, and 2.5 units/μl of Moloney murine leukemia virus RT in a total volume of 20 μl. Specific priming using 0.8 μM EGFR 4 primer was also compared with random priming. Specific priming was found to be at least as sensitive as random priming and was used in some assays to confirm EGFR positivity. After total RNA addition, the samples were incubated for 10 min at room temperature, followed by 15 min at 42°C (reverse transcription reaction), 5 min at 99°C (denaturation), and then maintained at 5°C for up to 2 h before proceeding with the PCR reaction.

The PCR reaction was performed by dilution of the 20-μl RT reaction mixture to a total volume of 100 μl by the addition of the following components to the indicated final concentration: MgCl₂ (2 mM), PCR buffer II (1×), AmpliTaq DNA polymerase (2.5 units/100 μl), and outer primers EGFR 1 and 4 (0.8 μM each primer). The PCR amplification was conducted on a GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer) with initial denaturation at 95°C for 1 min and 45 s, followed by 40 cycles using this cycling profile: denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 72°C for 60 s. A final extension step at 72°C for 7 min completed the reaction. Nested PCR was performed by adding 5 μl of the outer PCR product mixture to 95 μl of a nested reaction mix, which was identical to that used in outer PCR, except 0.8 μM of each nested primer EGFR 2 and 3 (Fig. 1) were added. Nested PCR was conducted for 40 cycles with the same thermal cycling profile as the outer PCR reaction.

Negative control reactions containing RNA without RT and reactions containing RT without RNA were run in parallel along with positive control reactions containing cell line RNA. Outer and nested PCR reactions were performed at separate sites, and additional precautions were taken to minimize the possibility of PCR carryover, including changing gloves after every transfer of outer PCR products to the nested PCR reaction mixture.

Aliquots of 20 μl of the PCR products were electrophoresed on 2% agarose gels containing ethidium bromide for 1–1.5 h and visualized on a UV transilluminator and photographed. The 100-bp DNA ladder used for molecular markers contained a 600-bp band two to three times brighter than the bands of other sizes (Life Technologies, Inc.). Outer PCR with EGFR 1 and 4 primers produced a 1086-bp product, and nested PCR with EGFR 2 and 3 primers yielded a 1027-bp product. All positive EGFR PCR results were confirmed by repeating the assay using another aliquot of total RNA. If the initial positive result could not be repeated, the result was considered negative.

RNA integrity was confirmed using human β₂-microglobulin primers that spanned two introns (1858 bases spanned) and generated a 536-bp product (Fig. 1; Ref. 26). A single round of RT-PCR was performed as described for EGFR, except that β₂-microglobulin primers (0.2 μM each) were used. Thermal cycling consisted of initial denaturation at 95°C for 1 min and 45 s, followed by 35 cycles using this profile: denaturation at 95°C for 15 s, then annealing and extension at 60°C for 30 s. A final extension step at 72°C for 7 min completed the reaction. Samples that did not demonstrate an intact β₂-microglobulin band were judged degraded and were not included in the analysis.
Statistical analysis was performed using the two-sided Fisher’s Exact test for comparing the number of EGFR-positive cases in the control and metastatic breast cancer patient groups.

Southern Blotting. Specificity of the PCR reaction was determined using Southern blot gel transfer, followed by hybridization. Briefly, after electrophoresis of the nested PCR products, the gel was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 45 min and then put in neutralizing solution (1 M Tris, 1.5 M NaCl, pH 7.4). Downward transfer of the gel to the Nytran membrane (Schleicher and Schuell, Keene, NH) was performed overnight, and the membrane baked at 80°C for 2 h. The membrane was prehybridized at 42°C for 2 h and hybridized at 50°C for 16 h with a 32P-labeled synthetic oligoprobe (Fig. 1). The EGFR probe was end-labeled to a specific activity of 2 x 10^6 cpm/ml using [γ-32P]ATP (3000 Ci/mmol; DuPont New England Nuclear, Boston, MA). After a series of washes with a final stringency of 0.1 X SSC and 0.1% SDS at 50°C, the membrane was exposed to Kodak XAR film with intensifying screens at room temperature for 4 h.

RESULTS

RT-PCR Detection and Sensitivity in Human Cell Lines. The EGFR oligonucleotide primers were designed to span EGFR introns 1 to 7 to avoid amplification of genomic DNA (Fig. 1). The RT-PCR assay detected EGFR bands in total RNA extracted from the following human cell lines: A431 epidermoid carcinoma (Fig. 2), SKBR3 (Fig. 2), MCF-7 and Hs578T breast cancer (Fig. 3A), and also BxPC-3 pancreatic cancer, WiDr colon cancer, and SKOV3 ovarian cancer (data not shown). When RT was omitted from the RT reaction, no bands were detected in any of the cell lines (Fig. 3A. -RT lanes). This was expected because the primers were designed internal to exons 1 and 8, containing greater than 30 kb of introns (Fig. 1). Therefore any contaminating genomic DNA in the total RNA preparations would not be amplified. The sensitivity of the RT-PCR assay was determined by diluting A431 oligonucleotide sequence (5'-3')

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5'-3')</th>
<th>Location (5'-3')</th>
<th>Strand</th>
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<td>EGFR 1</td>
<td>GCC GAC GCC CAC AAC CAC</td>
<td>100-117</td>
<td>Sense</td>
</tr>
<tr>
<td>EGFR 2</td>
<td>GCC CCT GAC TCC GTC CAG TAT TG</td>
<td>127-149</td>
<td>Sense</td>
</tr>
<tr>
<td>EGFR 3</td>
<td>GCC CTC TCC CCT CCA TCT CAT AGC</td>
<td>1153-1130</td>
<td>Antisense</td>
</tr>
<tr>
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<td>TCC CCG AAG AGC TGC GTC CGG GTC</td>
<td>151-180</td>
<td>Antisense</td>
</tr>
<tr>
<td>B2-M 1</td>
<td>AGC AGA GAA TGG AAA GTC AAA</td>
<td>Sense</td>
<td></td>
</tr>
<tr>
<td>B2-M 2</td>
<td>TGT TGA TGT TGG ATA AGA GAA T</td>
<td>Antisense</td>
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</table>

Fig. 2 Sensitivity of nested RT-PCR for EGFR using A431 RNA. The indicated concentration of A431 total RNA was diluted into 1 µg of negative control 7 total RNA (C7). M, 100-bp DNA ladder; N, no RNA; pg, picogram; fg, femtogram; ag, attogram.
EGFR bands (Fig. 4A). This difference was statistically significant when compared with the control group (P = 0.03). Patient 117 initially tested positive for EGFR (Fig. 4A), but subsequent re-assay failed to amplify the EGFR RNA, and this patient was therefore considered negative. This patient had the only initial positive result that could not be replicated. Non-repeatability of an initial RT-PCR positive result has been reported previously in breast cancer and was attributed to either sub-threshold transcript occurrence or PCR carryover (10). EGFR mRNA was not detected in the blood from 6 patients with local recurrence of breast cancer or in 13 patients receiving adjuvant therapy (data not shown). Southern blot analysis using an internal EGFR oligonucleotide probe confirmed the identity of the 1027-bp band from the four EGFR RT-PCR-positive metastatic breast cancer patients and the A431 cell line but detected nothing from the RNA from the healthy control individuals (Fig. 4B).

An analysis of possible treatment effects of chemotherapy or endocrine therapy was made on the metastatic breast cancer patients. All of these patients had clinical evidence of progressive disease at the time their blood was drawn. Three of the four EGFR RT-PCR-positive patients were untreated for at least 2 months prior to blood collection. One of these four patients was progressing while receiving chemotherapy (Navelbine). Of the 14 patients in the EGFR RT-PCR-negative patient group, 6 were untreated for a period of at least 2 months prior to blood drawing; 5 were being treated with hormone therapy, and 3 were receiving chemotherapy. Therefore, the EGFR mRNA detected in the blood from the four metastatic patients was not a treatment-induced phenomenon.

The influence of the site of metastasis on the RT-PCR results was also evaluated. All four of the RT-PCR-positive patients had visceral sites of metastases, whereas only 6 of the 14 RT-PCR-negative patients (43%) had visceral metastases (Table 1).

**DISCUSSION**

In this report, we describe the development of a sensitive RT-PCR assay using EGFR for the detection of cancer cells in the blood of patients with breast cancer. This assay detected EGFR-positive RNA in 4 of 18 (22%) patients with metastatic breast cancer, 0 of 6 patients with localized breast cancer, and 0 of 13 patients receiving adjuvant therapy after removal of the primary breast tumor. This frequency of RT-PCR-positive cases (22%) is indicative of circulating micrometastases in metastatic breast cancer patients is almost identical to that in reports published previously using other markers. Using cytokeratin 19, 4 of 19 (21%) metastatic breast cancer patients were RT-PCR positive (10). Using maspin, a tumor suppressor gene coding for a serine protease inhibitor, 4 of 20 (20%) patients were RT-PCR positive (15). We have also shown that RT-PCR positivity for EGFR was not a treatment-related phenomenon. Also, although our patient sample size was not large, RT-PCR positivity was associated to a greater degree with visceral sites of metastases, which are known to confer a worse prognosis. In our study, the lack of EGFR RT-PCR-positive cases in breast cancer patients without metastatic disease is also consistent with previous studies (10, 15). Lack of detection of circulating cancer cells in early-stage breast cancer may be due to the intermittent hema-
The use of RT-PCR for detection of circulating micrometastases offers a number of advantages compared with cytological and IHC techniques. Both techniques suffer from poor sensitivity, and in IHC, the antibodies used have some degree of false positivity (7). RT-PCR assays generally exhibit at least a 10-fold increase in sensitivity compared with IHC (7). The increased sensitivity and specificity of RT-PCR procedures for the detection of circulating micrometastases has led to the conclusion that these assays could have a profound impact on cancer therapy (7). In prostate cancer, the use of prostate-specific antigen RT-PCR is on the verge of clinical utility; it may play a role in staging of patient tumors before radical prostatectomy, in the serial follow-up of patients after treatment, and in improving the detection of lymph node metastasis (28). However, the development of RT-PCR for detecting micrometastasis in other cancers, including breast cancer (20), is not as advanced.

The use of EGFR as a detector molecule for RT-PCR of circulating micrometastases may have several advantages over those reported previously. The existence of a processed pseudogene for cytokeratins (19) has made interpretation of results unreliable in some studies (14, 18). In addition, the presence of illegitimate transcription of cytokeratins in hematopoietic cells has raised concerns about nonspecificity (16). We have encountered no evidence for pseudogene contamination using EGFR, and no illegitimate transcription was detected in the blood from 23 control individuals. In addition, unlike maspin, which is down-regulated during malignant progression (29), EGFR remains overexpressed in human breast cancers and may confer a worse prognosis (22).

A possible application for this EGFR RT-PCR assay could be monitoring the effectiveness of purging of breast cancer cells in patients undergoing high-dose chemotherapy and autologous

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**Table 1** RT-PCR results by site of metastatic breast cancer

<table>
<thead>
<tr>
<th>Metastatic site</th>
<th>RT-PCR status of blood RNA</th>
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<tr>
<td></td>
<td>EGFR-positive</td>
</tr>
<tr>
<td>Visceral</td>
<td>4</td>
</tr>
<tr>
<td>Bone only</td>
<td>0</td>
</tr>
<tr>
<td>Soft tissue/lymph nodes</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
</tr>
</tbody>
</table>

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1 K. Leitzel and A. Lipton, unpublished observation.
bone marrow transplantation. Previous studies have reported that the presence of cytokeratin 19 positivity in the bone marrow is associated with a poor prognosis (12). Because nested cytokeratin PCR for cytokeratin 19 has shown false positives in negative controls (16), the use of EGFR alone or in combination with cytokeratin 19 may be advantageous to evaluate purging efficiency and outcome for these patients (27).

The EGFR has been the target of intensive efforts in cancer therapy. The development of human:murine chimeric monoclonal antibodies to the extracellular domain of the EGFR has shown potential, either alone or in combination with chemotherapy (30). Further modifications of these antibodies with toxins (31) or endowing them with bispecific binding capacity (32) may enhance this approach. Another promising target is the intracellular tyrosine kinase domain of the EGFR. A number of tyrosine kinase inhibitors have been developed for inhibition of the activated EGFR tyrosine kinase (33, 34). Combination treatment using tyrosine kinase inhibitors and monoclonal antibodies to the EGFR have been reported recently to cause enhanced growth inhibition of the A431 cell line (35). Another recent approach has been the development of antisense oligonucleotides that inhibit EGFR expression and cell growth (36, 37). Regardless of the mode of EGFR-targeted therapy, the ability to select cancer patients by using RT-PCR with EGFR for the detection of circulating micrometastases could increase the possibility of treating patients most likely to benefit from these therapies.

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Detection of cancer cells in peripheral blood of breast cancer patients using reverse transcription-polymerase chain reaction for epidermal growth factor receptor.

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