Application of a Multigene Reverse Transcription-PCR Assay for Detection of Mammaglobin and Complementary Transcribed Genes in Breast Cancer Lymph Nodes

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Background: Mammaglobin mRNA expression is found in 70–80% of primary and metastatic breast tumor biopsies. The potential breast tumor markers B305D, B726P, and γ-aminobutyrate type A receptor π subunit (GABAπ) complement the expression of mammaglobin. Collectively the expression profile of these four genes could be used as a diagnostic and prognostic indicator for breast cancer.

Methods: A multigene reverse transcription-PCR (RT-PCR) assay was established to detect the expression of mammaglobin, GABAπ, B305D, and B726P simultaneously. Specific primers and TaqMan® probes were used to analyze combined mRNA expression profiles in primary breast tumors and metastatic lymph node specimens.

Results: The multigene RT-PCR assay detected substantial expression signals in 27 of 27 primary tumor and 50 of 50 metastatic breast lymph node samples. Specificity studies demonstrated no significant expression signal in 27 non-breast cancer lymph nodes, in 22 various healthy tissue samples, or in 14 colon tumor samples.

Conclusion: The novel RT-PCR-based assay described here provides a sensitive detection system for disseminated breast tumor cells in lymph nodes. In addition, this multigene assay could also be used to test peripheral blood and bone marrow samples. © 2002 American Association for Clinical Chemistry

Mammaglobin, a highly glycosylated low-molecular-weight protein, is a homolog of rabbit uteroglobin and rat steroid-binding protein subunit C3 (1, 2) and is a member of the uteroglobin family with yet unknown functions. The known human family members [mammaglobin, lipophilin A and B, mammaglobin B (lipophilin C, lacryglobin), Clara cell 10-kDa protein] are localized in a dense cluster on chromosome 11q12.2 (3). Mammaglobin mRNA expression of has been described in 70–80% of primary and metastatic breast tumor biopsies (1, 4–7). Furthermore, the mammaglobin gene has been used as a marker to detect circulating mammary carcinoma cells in peripheral blood by reverse transcription-PCR (RT-PCR)1.

Although mammaglobin is a promising tumor marker, it is not universally expressed in breast cancers because these tumors are highly heterogeneous by gene expression profiling. Therefore, additional markers are needed for development of a sensitive assay to detect malignant breast cancer cells. Three genes, B305D, γ-aminobutyrate type A receptor π subunit (GABAπ), and B726P, have been identified by genetic subtraction, differential display, DNA microarray analysis, and real-time PCR as potential therapeutic and diagnostic targets in breast cancer. Recently we reported the complementary expression profile of these candidate genes in combination with mammaglobin in breast carcinomas (7). B305D is a novel gene that is located on chromosome 21q11.1, c1 region (AP001465.1). B305D cDNA has been isolated by differen-

1 Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; GABAπ, γ-aminobutyrate type A receptor π subunit; FAM, 6-carboxyfluorescein; and TAMRA, 6-carboxytetramethylrhodamine.
tial display RT-PCR, and its sequence predicts a type II membrane protein.

The known gene GABA_{\alpha} was recovered by PCR subtraction and found to be inversely expressed compared with B305D. GABA_{\alpha} (U95367) belongs to the GABA_{\alpha} receptor family. Unlike other GABA_{\alpha} receptors, which are typically expressed in neuronal tissues, GABA_{\alpha} shows low expression in lung, thymus, and prostate tissues and higher expression in the uterus (9). The cDNA designated as B726P (AL357148) was derived from PCR subtraction. B726P is a novel gene located on chromosome 10 with several different putative open reading frames yielded by mRNA splicing. One of these splice forms has recently been identified (10) by use of reactivity with autologous breast cancer patient sera and referred to as NY-BR-1.

In the present study we established a real-time multigene RT-PCR assay to simultaneously monitor the expression of these four breast cancer markers. The sensitivity and specificity of this assay were investigated using numerous primary breast tumors, metastatic lymph node, and healthy tissues.

**Materials and Methods**

**TISSUE SAMPLES**
Primary breast cancer and healthy tumor lymph node tissue samples were kindly provided by Dr. Elizabeth Repasky (Roswell Park Cancer Institute, Buffalo, NY) and Dr. Roberto Badaro (University of Bahia, Salvador, Brazil). Tissue samples were also obtained from National Disease Research Interchange. Lymph node cDNA samples were kindly provided by Dr. Michael Mitas (Medical University of South Carolina, Department of Surgery, Charleston, SC) and Dr. Timothy P. Fleming (Washington University School of Medicine, St. Louis, MO).

**RNA ISOLATION and cDNA PREPARATION**
Total RNA was extracted from liquid-nitrogen-frozen tissue samples by homogenization in Trizol reagent (Invitrogen), and cDNA was prepared using oligo(dT) primer (Boehringer Mannheim) with Superscript\textsuperscript{TM} II reverse transcriptase (Invitrogen) for 60 min at 42 °C.

**PCR ASSAY**
A multigene real-time PCR assay was developed to detect the expression of the mammaglobin gene, GABA_{\alpha}, B305D, and B726P simultaneously. The expression of single genes and their combined expression was measured by quantitative real-time PCR using the ABI7700 Prism\textsuperscript{TM} sequence detection system (Applied Biosystems). Specific primers and 6-carboxyfluorescein (FAM)-labeled TaqMan\textsuperscript{®} probes were used in combination. Primers were designed to span exon-exon junctions to exclude genomic DNA from amplification. Primer concentrations that limited the reporter signal without decreasing the detected threshold cycle to avoid competition in the multigene PCR were determined.

The forward primer for mammaglobin was 5'-tgccata-

**B726P** and the reverse primer was 5'-tgcataatatattgcagaacatc-3', both used at 100 nmol/L. The primer sequences for GABA_{\alpha} were 5'-caatatttgagtggagggccgct-3' (forward) and 5'-gctgtggggtgttactagtg-3' (reverse), used at 300 nmol/L and 50 nmol/L, respectively. For B305D amplification, 300 nmol/L forward primer 5'-tcgtaaagccgttaactg-3' and primer 50 nmol/L reverse primer 5'-cagactgtctgggttcct-3' were used. For B726P, 100 nmol/L each of forward primer 5'-gagacccgtcaatgataagggc-3' and reverse primer 5'-atatagctagattacatacactc-3' were added to the PCR mixture. Actin expression was measured in separate reactions and used to normalize expression values; 300 nmol/L each of forward primer 5'-actggaagcggagggagca-3' and reverse primer 5'-eggccacatttgtaacctg-3' were used for actin amplification. For real-time detection, 4 pmol of each gene-specific TaqMan probe with the following sequences were used:

- Mammaglobin: FAM-5'-tctaaccacggatagaccttaga-3'–6-carboxytetramethylrhodamine (TAMRA) GABA_{\alpha}:
  - FAM-5'-cattttcagagtagaatacgctacacta-3'–TAMRA B305D:
  - FAM-5'-atacaaaaacagcatggctctaccact-3'–TAMRA B726P:
  - FAM-5'-ccccatcatgataaccaacagggagagct-3'–TAMRA Actin: FAM-5'-cagctggctgtgagcagctccc-3'–TAMRA

Forty PCR cycles were performed with TaqMan 1000 Rxn PCR Core Reagents (part no. 430 4439; Applied Biosystems) using 0.0375 U/\mu L TaqGold; 1× Buffer A; 5 mM MgCl\textsubscript{2}; 0.2 mM each of dCTP, dATP, and dGTP; 0.4 mM dUTP; 0.01 U/\mu L AmpErase UNG; 80 nL/\mu L glyc erol; 0.5 nL/\mu L gelatin; and 0.1 nL/\mu L Tween 20. PCR conditions were as follows: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s followed by 60 °C for 1 min and 68 °C for 1 min. For single-gene PCR, calibration curves were established using serial dilutions of plasmid DNA containing target gene cDNA sequence. For the multigene assay, combined copy numbers were determined by use of serial dilutions containing four plasmids to establish a calibration curve. TaqMan SDS analysis software was used to determine calibration curves and to calculate copy numbers. Final copy numbers were determined as mean values of triplicate PCR reactions and normalized per 1000 pg of actin. The mean variation within triplicates was 21% (range, 3–35%).

**ROC CURVE ANALYSIS**
Rockit 0.9B Beta Version software (Dr. Charles E. Metz, Department of Radiology, The University of Chicago, Chicago, IL; http://www-radiology.uchicago.edu/krli/toppage11.htm) was used to analyze expression data obtained from pathology-positive breast cancer and healthy lymph node specimens. Detected copy numbers in lymph node samples for the mammaglobin, B305D, B726P, and GABA_{\alpha} assays or the multigene RT-PCR assay, respectively, were processed for the 27 actually
negative and the 50 actually positive cases. The generated output of true-positive fractions and the corresponding false-positive fractions was used for the construction of ROC curves as sensitivity and (1 – specificity).

**Results**

A panel of 27 primary breast tumor samples was used to compare the multigene real-time PCR assay with single-gene PCR. The multigene assay, which simultaneously detected the mammaglobin, B305D, B726P, and GABA genes, yielded a large expression signal for 27 of 27 tumor samples (Fig. 1B) compared with mammaglobin expression alone, which detected 17 of these samples (Fig. 1A). Table 1 further demonstrates the transcriptional complementation of the four target genes, with B305D being highly expressed in 16, GABA in 10, and B726P in 14 tumor specimens. All samples tested were detected positive with a combined expression signal by the multigene assay.

The expression profiles of B305D and GABA in the breast tumor samples showed an inverse relationship that was complementary. This led to the detection of 24 of 27 samples.
primary breast tumor samples based on the expression of these two genes alone. B726P expression showed an independent but additive profile compared with the other markers.

We then tested the cDNA from 50 pathology-positive metastatic breast cancer lymph node samples and 27 healthy or nonrelevant disease lymph node samples, respectively. All 50 metastatic breast cancer samples were detected with positive expression signals (Fig. 2), whereas nonbreast cancer-containing lymph node specimens showed no significant signals. Table 2 illustrates the complementary expression profiles of the four candidate genes in the 77 lymph node specimens tested.

Substantial mammaglobin expression alone was detected in 40 metastatic breast cancer lymph node samples, ranging from 19.7 to 4.1 × 10⁶ copies/1000 pg of actin. High expression of B305D was found in 36 (5–5 × 10⁴ copies/1000 pg of actin), of GABAα in 15 (11.7–830 copies/1000 pg of actin), and of B726P in 22 (7–4.9 × 10⁴ copies/1000 pg of actin) of 50 pathology-positive breast cancer lymph node samples. No large expression signals were detected by the mammaglobin, B305D, GABAα, B726P, or multigene RT-PCR assays in 24 healthy lymph node specimens, in 2 nodes containing melanoma, or in 1 node with evidence of lymphoma.

The multigene real-time PCR assay detected a positive signal for all 50 metastatic breast cancer lymph node specimens, ranging from 17.2 to 1.9 × 10⁴ copies/1000 pg of actin. Similar to primary tumor samples, two genes in particular, B305D and GABAα, added to the diagnostic sensitivity of mammaglobin detection in lymph node analysis. A combination of these three genes detected 50 of 50 lymph node breast metastases and 26 of 27 primary breast tumors evaluated. The inclusion of B726P enabled the detection of one primary tumor specimen. Moreover, parallel detection of the four genes enhanced the detection sensitivity for samples with low single-gene expression.

To assess the specificity of the multigene PCR assay described here, we tested a cDNA panel containing 14 colon tumor and 22 healthy tissue samples. Expression signals were detected in 0 of 14 colon tumors and in 4 of 22 healthy tissue samples (Table 2). However, the borderline signals detected in the four healthy tissue samples (esophagus, skin, trachea, and salivary gland) were only 2–6 copies/1000 pg of actin and were presumably attributable to low-level expression of mammaglobin in skin and of GABAα and mammaglobin in salivary gland, trachea, and esophagus. No background expression was detected in bone marrow and peripheral mononuclear cells.

To compare the diagnostic performance of single markers and multimarker detection in lymph node analysis, we analyzed the expression data using Rockit 0.9B Beta Version software. ROC curves were established by plotting sensitivity vs (1 – specificity) to assess the accuracy of each assay (Fig. 3). The software was used to calculate ROC curve sensitivities and specificities and the corresponding area under the curve. The area under the ROC curve indicates the performance of an assay to separate the group being tested into actually negative and actually positive cases. A perfect test is represented by an area of 1, an excellent test by 0.9–1, and a good test by 0.8–0.9; 0.7–0.9 indicates moderate accuracy. Because of the perfect decision performance of the multigene assay, no actual curve could be constructed. ROC analysis of the multigene data produced an area value under curve of 1. The best performance for a single-gene expression assay was achieved by mammaglobin, with a ROC curve area value of 0.92. B305D expression data produced an area value of 0.84, which indicates good test performance. B726P RT-PCR, with an area under the curve of 0.78, and GABAα RT-PCR, with an area under the curve of 0.77, can be categorized as assays with moderate accuracies.

**Discussion**

Lymph node staging provides the most important prognostic indicator in breast cancer (11). The presence of metastases in lymph nodes correlates directly to the risk of disease recurrence and patient survival. Convention-
ally, axillary dissection is used to collect lymph nodes for analysis. As an alternative, sentinel lymphadenectomy has been discussed as a less invasive strategy to identify lymphatic spread of metastatic cancer cells (5, 12, 13). Nodal metastases are identified by staining (hematoxylin or eosin) or immunohistochemical analysis for cytokeratin proteins (14). Inadequate sectioning in lymph node analysis can produce false-negative results by missing small metastatic foci. Approximately 30% of the patients diagnosed with pathologically negative lymph nodes develop recurrent disease. Because undetected micrometastases can be considered as a potential source for relapse of disease, more sensitive techniques for lymph node analysis have been discussed (5, 15–17). Molecular diagnostic approaches are potentially of higher sensitivity than immunohistochemistry. However, the application of RT-PCR for breast cancer cell detection is hampered by the lack of specific marker genes. Cytokeratins as epithelial cell marker genes have shown limited specificity for breast cancer cell detection (14, 18). Other potential tumor markers, in particular carcinoembryonic antigen, mucin 1, and mamaglobin, demonstrate higher specificity, but identify only a subset of tumor specimens (4, 5, 18, 19). No specific universal tumor marker has been identified to date because of the biological heterogeneity of breast carcinomas (20).

In this study we demonstrate the use of complementary expressed breast cancer genes for the development of a sensitive and specific RT-PCR assay to detect metastatic breast cancer cells. The multimarker assay described here increases the probability of detecting disseminated tumor cells and also provides the potential to characterize micrometastases on a molecular level. This could lead to improvements in prognosis and monitoring of disease, and possibly to individualized therapeutic strategies in the future.

For the future, intraoperative analysis of sentinel lymph nodes based on RT-PCR might even be possible. As a result of improved screening approaches, early detection of breast tumors with small diameters is increasing. Because the incidence of lymph node metastases is related to tumor size, for small tumors the value of routine axillary lymph node dissection, in consideration of the possibility of overtreatment and consequent morbidity, is a matter of much debate (21–23). The development of rapid quantitative RT-PCR protocols might provide the platform to realize highly sensitive intraoperative analysis of sentinel lymph nodes (24) and to gain prognostic information about the necessity of axillary dissection for disease control.

This study focused on lymph node analysis to evaluate the specificity and sensitivity of the assay. However, the multimarker assay has potential applications in various

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<th>Lymph node specimens</th>
<th>Metastatic breast cancer</th>
<th>Melanoma/ Lymphoma</th>
<th>Healthy</th>
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<td>Mammaglobin</td>
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<td>B305D</td>
<td>36/50</td>
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<td>GABA/H9266</td>
<td>15/50</td>
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<td>B726P</td>
<td>22/50</td>
<td>0/3</td>
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<tr>
<td>Multigene assay</td>
<td>50/50</td>
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<th>Colon tumor specimens</th>
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<th>Table 2. Positive expression signals for single-gene and/or multigene analyses in lymph node specimens, in colon tumor specimens, and in healthy tissues.</th>
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| a Two melanoma and one lymphoma lymph node specimen. |
| b Two healthy colon, peripheral blood mononuclear cells, bone marrow, heart, brain, pancreas, lung, liver, skin, kidney, spinal cord, salivary gland, small intestine, stomach, trachea, adrenal gland, aorta, skeletal muscle, bone, esophagus, bladder. |
| c A low expression signal was detected in esophagus, skin, trachea, and salivary gland cDNA (2–6 copies/1000 pg of actin). |
tissues as a sensitive tool to detect breast tumor cells. Recently, we reported initial studies for these four genes individually (7) and in combination (25) to detect circulating breast tumor cells in peripheral blood samples.

Metastatic cells in bone marrow may also provide a high predictive value (26) for relapse of disease, independent of lymph node status. In addition, detection of cytokeratin-positive cells in the bone marrow of early-stage tumors has been suggested as a potential marker for early diagnosis of tumor dissemination (27). RT-PCR for mammaglobin has been shown to successfully detect bone marrow micrometastases (28). Mammaglobin gene expression is also a useful marker for detection of tumor cell contamination in leukaphereses, with potential application in autologous stem cell transplantation protocols for high-dose chemotherapy programs (29). Because of the increased sensitivity provided by the multigene assay compared with mammaglobin alone, we conclude that the method described here will be useful for the evaluation of peripheral blood, bone marrow, and leukapheresis samples for disseminated breast cancer cells.

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