

Molecular Detection of Breast Cancer Cells in the Peripheral Blood of Advanced-Stage Breast Cancer Patients Using Multimarker Real-Time Reverse Transcription-Polymerase Chain Reaction and a Novel Porous Barrier Density Gradient Centrifugation Technology

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ABSTRACT

Purpose: The goal of this study was to develop a molecular diagnostic assay to detect circulating breast cancer cells in the peripheral blood for the purpose of staging breast cancer. Our aim was to make available an assay that was not limited by the low concentration of circulating breast cancer cells and the background gene expression that is typically found in peripheral blood.

Experimental Design: In this study, we investigated the ability of two new technologies to significantly enhance the quantification of gene expression in the peripheral blood: enrichment by a novel porous barrier density gradient centrifugation technology; and multimarker real-time reverse transcription-PCR (RT-PCR).

Results: Using fluorescence-labeled breast cancer cells and flow cytometry, we show that processing peripheral blood by porous barrier density gradient centrifugation results in a 300-fold enrichment of breast cancer cells. Real-time RT-PCR analysis confirmed a concomitant reduction in background expression of the *CK19* and *MUC1* genes after enrichment. In a pilot study, porous barrier density gradient centrifugation and multimarker real-time RT-PCR enabled our laboratory to detect breast cancer-associated gene overexpression in 13 of 20 (65%) stage IV breast cancer

patients. Nine of these 14 patients overexpressed three or more markers.

Conclusions: These results confirm the promise of such a molecular diagnostic assay and suggest that additional studies are needed to precisely define the clinical relevance.

INTRODUCTION

The goal of breast cancer staging is to classify patients by the extent of disease into groups with similar clinical outcomes. Staging facilitates patient management, allowing clinicians to tailor therapies to individual patients. Unfortunately, current staging for breast cancer is invasive, expensive, and lacks sensitivity. Breast cancer staging is based on the AJCC² tumor-node-metastasis (TNM) system, which relies heavily on the pathological evaluation of the primary tumor (T), regional lymph nodes (N), and distant metastases (M). Within this framework, the presence of disease in the ALNs is an important prognostic indicator for breast cancer patients (1). As a result, staging for newly diagnosed clinical stage I and II breast cancer patients typically includes an ipsilateral ALN dissection or sentinel lymph node biopsy. Despite the application of this relatively invasive and expensive staging procedure, up to 30% of node-negative patients ultimately develop recurrent disease (2). This suggests that the tissue being sampled and/or the means of disease detection are inadequate. Additionally, the detection of distant metastatic disease in breast cancer patients is also problematic. Presently, clinicians will obtain a staging evaluation that includes computed tomography of the chest, abdomen, and pelvis and a nuclear medicine bone scan as the clinical situation indicates. However, such an evaluation is limited by expense and low sensitivity.

The recent identification of genes overexpressed in breast cancer, combined with advances in molecular biology, provides the opportunity to establish more sensitive, specific, and cost-effective ways of identifying metastatic disease (3–14). Among the current possibilities, one of the most compelling is the development of a sensitive molecular diagnostic assay for the detection of breast cancer in the peripheral blood. Such an assay could potentially replace invasive procedures such as ALN dissection and/or be used for breast cancer screening and monitoring treatment responses. Unfortunately, molecular analysis

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² The abbreviations used are: AJCC, American Joint Committee on Cancer; RT-PCR, reverse transcription-PCR; ALN, axillary lymph node; FBS, fetal bovine serum; TP, ThinPrep; ICC, immunocytochemistry; CFSE, 5,6-carboxy fluorescein succinimidyl ester.

of peripheral blood has proven more challenging than that of other tissue compartments. mRNA is relatively unstable in peripheral blood, and the presence of hemoglobin can inhibit the PCR reaction. Other issues include the relatively low concentration of circulating breast cancer cells and the high level of background gene expression in peripheral blood. Two new technologies, however, should permit the rational development of a molecular diagnostic assay for the detection of breast cancer in the peripheral blood: real-time RT-PCR; and novel density gradient centrifugation techniques for enrichment of breast cancer cells from peripheral blood (15).

The current standard for isolation of tumor cells from peripheral blood is Ficoll density gradient centrifugation. A major limitation of this technique is concomitant isolation of lymphocytes, cells that often express low levels of genes that serve as surrogate markers for cancer cells. In this study, we rigorously tested the ability of a novel porous barrier density gradient centrifugation technology to enrich breast cancer cells. We showed a dramatic enrichment using flow cytometry and molecular assays. Furthermore, the combination of enrichment technologies and real-time RT-PCR allows the sensitive detection of breast cancer-associated gene overexpression in the peripheral blood of stage IV breast cancer patients.

MATERIALS AND METHODS

Peripheral Blood Specimens. These studies were approved by the Medical University of South Carolina Institutional Review Board, and informed consent was obtained from all patients enrolled. Twenty healthy volunteers and 20 stage IV breast cancer patients were enrolled between August 1, 2001 and October 31, 2001. All patients were staged according to the AJCC standard criteria using relevant clinical data. Peripheral blood specimens (20 ml) were collected in K₃ EDTA tubes (Vacutainer) and immediately placed on ice. In an effort to decrease the risk of epithelial cell contamination, the first 5 ml of peripheral blood collected were discarded. Samples were then processed using a novel porous barrier density gradient centrifugation media (OncoQuick; Hexal Gentech, Holzkirchen, Germany) per the manufacturer's instructions. Briefly, precooled 50-ml centrifugation tubes containing 15 ml of separation medium below a porous barrier were filled with 20 ml of peripheral blood and centrifuged at 1600 × *g* for 20 min. The entire volume of the upper compartment was then collected and washed for 10 min at 200 × *g*. Pelleted cells were then further evaluated.

Cell Lines. All cell lines were obtained from American Type Culture Collection (Manassas, VA). Media and FBS were purchased from Life Technologies, Inc. (Rockville, MD). Cell lines and growth conditions were as follows: the breast cancer cell line MDA-361 was grown in Leibovitz's L-15 medium with 10% FBS in a non-CO₂ environment; and the SkBr3 breast cancer cell line was grown in McCoy's 5a medium with 1.5 mM L-glutamine, 3.0 g/liter glucose, 2.2 g/liter sodium bicarbonate, and 10% FBS.

ICC. Specimens were collected, washed in CytoLyt (Cytoc, Boston, MA) to lyse blood cells, and then resuspended in PreservCyt (Cytoc). Two TP slides were prepared and stained with Papanicolaou stain, and one slide was used for ICC. A

monoclonal antibody for cytokeratin (AE1/AE3) was used in conjunction with an automated immunostaining system (DAKO Autostainer; DAKO Cytomation, Carpinteria, CA) and a Nexus immunohistochemistry slide staining apparatus (Ventana Medical Systems Inc., Tucson, AZ). Immunostaining was performed with the avidin-biotin immunoperoxidase complex method of Hsu *et al.* (16). Briefly, the slides were incubated with primary antibody for 30 min and then incubated with secondary biotinylated antibody for 4 min. To visualize the antibody, the TP slide was treated with diaminobenzidine (0.05%) in 0.05 M Tris-HCl buffer (pH 7.8) with 0.03% H₂O₂ for 6 min and then washed in H₂O. The TP slide was counterstained with hematoxylin, dehydrated, cleared in xylene, and mounted in Permount.

mRNA Isolation and cDNA Synthesis. Poly(A) tail enriched mRNA was isolated from peripheral blood using Dynabeads mRNA Direct Micro Kit (Dyna, Oslo, Norway). Fifty percent of the final mRNA volume (12 μl) was reverse transcribed with 0.5 μg of oligo(dT)₁₂₋₁₆ and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in a reaction volume of 20 μl (10 min at 70°C, 50 min at 42°C, and 15 min at 70°C).

Real-Time RT-PCR. Primers used for real-time RT-PCR were as reported previously (17, 18) and are indicated in Table 1. Real-time RT-PCR was performed on a PE Biosystems Gene Amp 5700 Sequence Detection System (Foster City, CA). All reaction components were purchased from PE Biosystems. The standard reaction volume totaled 10 μl and contained 1× SYBR RT-PCR buffer; 3 mM MgCl₂; 0.2 mM each of dATP, dCTP, and dGTP; 0.4 mM dUTP; 0.25 unit of AmpliTaq Gold; 0.1 unit of AmpErase uracil-N-glycosylase erase enzyme; 0.35 μl of cDNA template; and 50–900 nM oligonucleotide primer. Initial steps of RT-PCR were 2 min at 50°C for uracil-N-glycosylase erase activation, followed by a 10-min incubation at 95°C. Cycles (*n* = 40) consisted of a 15-s denaturation phase at 95°C, followed by a 1-min annealing/extension at 60°C. The final phase was a 60°C incubation for 1 min. All reactions were performed in triplicate. The threshold for cycle-of-threshold (C_t) analyses of all samples was set at 0.5 relative fluorescence unit. Gene expression is quantitated in terms of the mean normalized gene expression, a ratio of the target gene to the reference gene, in this case *β2-microglobulin*. To do this, we have used a standard for the mathematical evaluation and analysis of real-time RT-PCR results recently published in *BioTechniques* (19). This publication outlines equations for calculating the mean normalized gene expression according to the following equation:

$$\text{MNE} = \frac{(E_{\text{ref}})^{\text{CT}_{\text{ref,mean}}}}{(E_{\text{target}})^{\text{CT}_{\text{target,mean}}}}$$

CFSE Labeling of MDA-361 Breast Cancer Cells. To assess the relative enrichment of breast cancer cells with porous barrier density gradient centrifugation (15), MDA-361 breast cancer cells were labeled with CFSE (Molecular Probes, Eugene, OR). Cells were suspended in 5 ml of CFSE stock solution and incubated at 37°C for 15 min. The cells were pelleted and resuspended in 25 ml of PBS and incubated for 25 min at 37°C.

Fig. 1 Selective enrichment of breast cancer cells with porous barrier density gradient centrifugation. MDA-361 cells were labeled with the fluorescent dye CFSE. Labeled cells were then spiked into peripheral blood obtained from healthy volunteers. Samples were then processed with porous barrier or Ficoll density gradient centrifugation. Forward-scatter versus side-scatter dot plots and CFSE histograms are shown. **A**, samples processed with Ficoll density gradient centrifugation. **B**, samples processed with porous barrier density gradient centrifugation (relative enrichment greater than 300-fold; see Table 1).

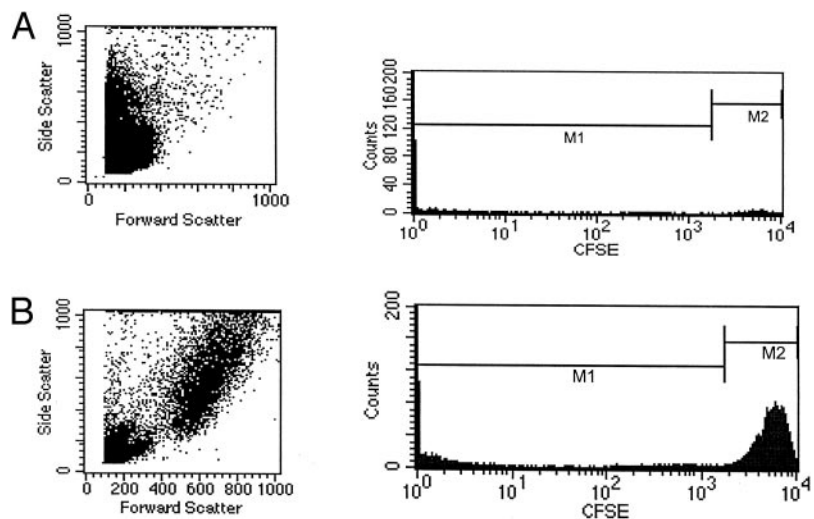


Table 1 Multimarker gene panel and primer pairs

Gene	Sequence of selected primer pair	Amplicon length	GenBank accession no.	Ref.
<i>MGLO</i>	F ^a 5'-GCCGTGTGAACCATGTGACTTT R 5'-CCAAATGCGGCATCTTCAAA	97	AB021288	This work
<i>PDEF</i>	F 5'-AGTGCTCAAGGACATCGAGACG R 5'-AGCCACTTCTGCACATTGCTG	90	AF071538	Mitas <i>et al.</i> , 2002 (18)
<i>MAM</i>	F 5'-CGGATGAAACTCTGAGCAATGT R 5'-CTGCAGTCTGTGAGCCAAAG	108	AF015224	Mitas <i>et al.</i> , 2001 (17)
<i>CK19</i>	F 5'-CATGAAAGCTGCCTTGGGAAGA R 5'-TGATTCTGCCGCTCACTATCAG	138	Y00503	Mitas <i>et al.</i> , 2001 (17)
<i>MUC1</i>	F 5'-ACCATCCTATGAGCGAGTACC R 5'-ACCATCCTATGAGCGAGTACC	107	J05581	Mitas <i>et al.</i> , 2001 (17)
<i>PIP</i>	F 5'-GCCAACAAAAGCTCAGGACAAC R 5'-GCAGTGACTTCGTCATTTGGAC	89	J03460	Mitas <i>et al.</i> , 2001 (17)
<i>MAMB</i>	F 5'-AGCAGTGTTCCTCAACCAGTC R 5'-TCTGAGCCAAACGCCTTG	126	AF071219	Mitas <i>et al.</i> , 2001 (17)

^a F, forward; R, reverse.

Cells were then washed in warmed PBS two times before spiking into normal peripheral blood specimens.

Breast Cancer Cell Micromanipulation. Certain spiking experiments required that 1, 10, or 100 MDA-361 cells be spiked into peripheral blood samples. This was accomplished under direct visualization with a dissecting microscope. Breast cancer cells were diluted until individual cells could be pipetted. Confirmation of cell acquisition was obtained by staining the source sample with trypan blue. These experiments were performed with the assistance of staff from the Hollings Cancer Center Transgenic Mouse Facility (Charleston, SC).

RESULTS

Porous Barrier Density Gradient Centrifugation Dramatically Enriches Breast Cancer Cells from Peripheral Blood. Porous barrier density gradient centrifugation is a novel technology designed to enrich cancer cells from peripheral blood (15). To assess the efficacy of this technology, MDA-361 breast cancer cells were labeled with the fluorescent dye CFSE and spiked into 15 ml of normal peripheral blood at

a concentration of 10,000 cells/ml. Samples were then processed with porous barrier density gradient centrifugation or Ficoll density gradient centrifugation. The absolute number of cells recovered was determined by manual count using a hemocytometer. Flow cytometry was then performed on the samples, and the percentage of breast cancer cells in each sample was determined using forward- and side-scatter characteristics and fluorescence intensity. The results of a typical experiment are represented in Fig. 1. The percentage of breast cancer cells after Ficoll density gradient centrifugation was 0.40%, but it was 39.42% after porous barrier density gradient centrifugation. Factoring in these percentages and the absolute number of cells recovered per sample, porous barrier density gradient centrifugation resulted in an enrichment of 300-fold (Table 1).

The flow cytometry results were reproduced with cytology. Breast cancer cells were spiked into normal peripheral blood as detailed above and then processed by porous barrier or Ficoll density gradient centrifugation. The specimens were then analyzed in a blinded fashion by a skilled cytopathologist, and the number of peripheral blood mononuclear cells and breast cancer

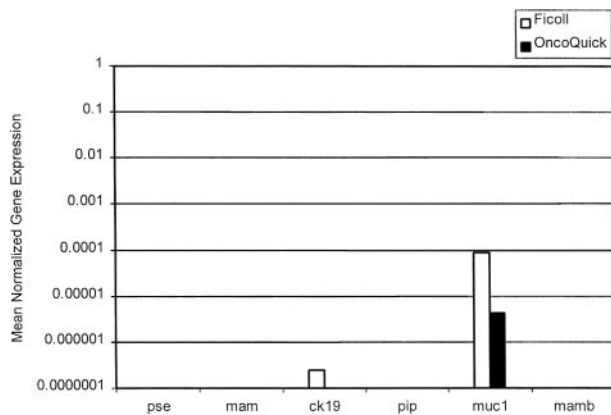


Fig. 2 Reduction of molecular background with porous barrier density gradient centrifugation. Peripheral blood from healthy volunteers was processed with porous barrier or Ficoll density gradient centrifugation. Multimarker real-time RT-PCR was performed, and gene expression was quantified based on mean normalized gene expression. With CK19 and MUC1, there is significant expression noted when peripheral blood is processed with Ficoll, but this is reduced or absent when blood is processed with porous barrier density gradient centrifugation.

cells was quantitated. Porous barrier density gradient centrifugation routinely reduced the number of peripheral mononuclear cells from a 15-ml sample to less than 500 cells. Recovery of breast cancer cells at spiking concentrations of 10–100 cells in 15 ml of peripheral blood ranged from 30% to 75%. When samples were processed with Ficoll density gradient separation, the large number of peripheral blood cells precluded the reliable identification of recovered breast cancer cells (data not shown).

Porous Barrier Density Gradient Centrifugation Significantly Reduces Background Gene Expression in Peripheral Blood. Conceptually, enrichment of breast cancer cells from peripheral blood can improve molecular analyses by two distinct mechanisms. First, enrichment can reduce background gene expression levels for molecular markers that are expressed in peripheral blood cells. Second, enrichment can enhance the signal from genes overexpressed by breast cancer cells. Previous studies have shown that low levels of *MUC1* and *CK19* are expressed in normal peripheral blood (20, 21). To address the first mechanism, reduction of background gene expression, samples of normal peripheral blood were processed with Ficoll and porous barrier density gradient centrifugation (Fig. 2). Samples were then analyzed with multimarker real-time RT-PCR. Background gene expression was significantly reduced after porous barrier density gradient centrifugation, particularly for the molecular markers *MUC1* and *CK19*.

Porous Barrier Density Gradient Centrifugation and Multimarker Real-Time RT-PCR Can Be Used to Detect Breast Cancer Gene Overexpression in the Peripheral Blood with Exquisite Sensitivity. To determine whether enrichment can enhance the signal from genes overexpressed by breast cancer cells, cell spiking experiments were performed. A fixed number of MDA-361 cells were spiked into 15 ml of normal peripheral blood (1, 10, 150, and 1500 cells/15 ml peripheral blood). Cell spiking was performed using a micromanipulation device to ensure accuracy. Samples were then processed with

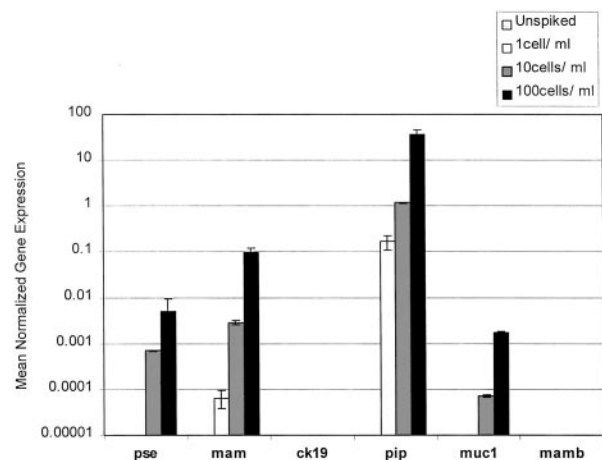


Fig. 3 Detection of gene overexpression in peripheral blood samples spiked with breast cancer cells. MDA-361 breast cancer cells were spiked into 15 ml of peripheral blood using a micromanipulation device at the concentrations indicated. Samples were then processed by porous barrier density gradient centrifugation and multimarker real-time RT-PCR. Results are expressed in terms of mean normalized gene expression.

porous barrier or Ficoll density gradient centrifugation and multimarker real-time RT-PCR. With porous barrier density centrifugation, overexpression of breast cancer-associated genes could be detected when as few as one breast cancer cell was spiked into 15 ml of peripheral blood (Fig. 3). This corresponds to a sensitivity of 1 breast cancer cell among 5×10^8 peripheral blood cells. Gene overexpression was consistently detected when 10 or more breast cancer cells were spiked into 15 ml of peripheral blood.

The Combination of Porous Barrier Density Gradient Centrifugation and Multimarker Real-Time RT-PCR Can Be Used for the Molecular Detection of Breast Cancer Cells in the Peripheral Blood of Stage IV Breast Cancer Patients.

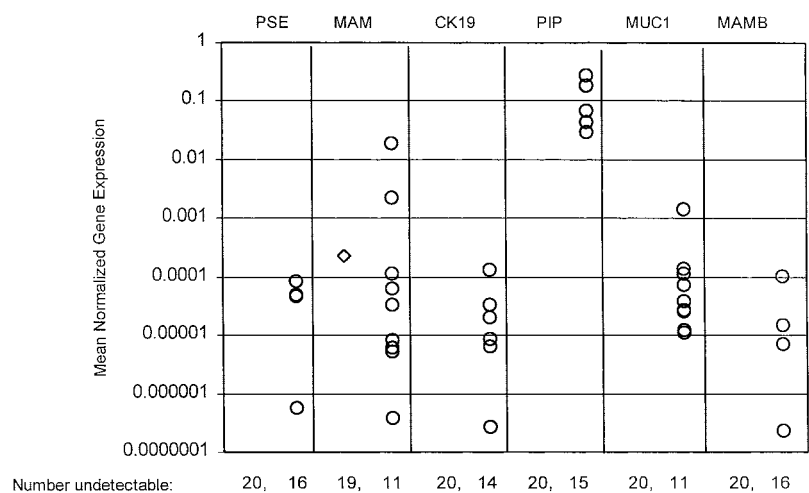
To assess the ability of porous barrier density gradient centrifugation and multimarker real-time RT-PCR to detect circulating breast cancer cells in the peripheral blood of breast cancer patients, peripheral blood from 20 healthy volunteers and 20 patients with stage IV breast cancer was obtained. The samples were enriched for breast cancer cells using porous barrier density gradient separation and then analyzed by real-time RT-PCR (40 cycles) using a previously defined multigene marker panel (Table 2; Ref. 17). Gene expression was quantitated by determining cycle threshold values (Fig. 4). Sixteen of the 20 stage IV patients were undergoing systemic chemotherapy at the time of participation. The average patient age was 53 ± 13 years. Sixty-five percent of patients ($n = 13$) had evidence of gene overexpression, with a mean overexpression of 3.0 molecular markers. The most frequently overexpressed genes were *mam* and *muc1* (69% both), followed by *CK19* (46%), *PIP* (38%), *PSE* (31%), and *mamb* (31%). Twenty percent of patients expressed one marker. Of the remaining patients, 25% expressed three markers, and 20% expressed four or more (Fig. 5). Of note, all patients naïve to chemotherapy ($n = 4$) had evidence of gene overexpression in the peripheral blood.

Table 2 Relative enrichment of breast cancer cells with porous barrier density gradient centrifugation

	Ficoll	Porous barrier
No. of breast cancer cells spiked into peripheral blood specimen	150,000	150,000
No. of mononuclear cells in peripheral blood specimen	1.1×10^8	1.1×10^8
Percentage of breast cancer cells based on cell counts	0.13%	0.13%
Total no. of cells recovered after enrichment	2.2×10^7	2.1×10^5
Percentage of breast cancer cells as determined by flow cytometry	0.4%	39.4%
Absolute no. of breast cancer cells recovered	8.8×10^4	8.3×10^4
Percentage of recovery	58.7%	55.2%
Relative enrichment	3-fold	303-fold
Absolute no. of mononuclear cells remaining after enrichment	2.19×10^7	1.3×10^5
Relative reduction in the no. of mononuclear cells	5-fold	846-fold

Gene expression in 20 healthy volunteers and 20 Stage IV breast cancer patients

Fig. 4 Detection of gene overexpression in stage IV breast cancer patients. Peripheral blood specimens were obtained from breast cancer patients with known stage IV disease. The specimens were processed with porous barrier density gradient centrifugation and multimarker real-time RT-PCR. Gene expression was expressed in terms of mean normalized gene expression. The individual genes are indicated at the top of the figure. Specimen analyses from healthy volunteers are indicated with a diamond on the left of each column. Specimen analyses from stage IV breast cancer patients are indicated with a circle on the right of each column. The number of healthy volunteers/patients where no gene expression was detected is indicated at the bottom of the figure.



DISCUSSION

Currently, breast cancer staging is based on the AJCC TNM system. The extent of regional disease is presently assessed by routine ipsilateral ALN sampling, a relatively invasive procedure. The presence of metastatic disease is determined using sophisticated, expensive diagnostic imaging studies that lack sensitivity. Thus, the development of a molecular diagnostic assay capable of detecting breast cancer-associated gene expression in the peripheral blood has the potential to vastly improve breast cancer staging and treatment (22). Although the literature currently supports molecular diagnostic assays in the detection of breast cancer cells in the peripheral blood of stage IV breast cancer patients (4, 20, 23–25), the successful development of such an assay is currently limited by two critical issues: the extremely low concentration of breast cancer cells in the peripheral blood; and the background expression of breast cancer-associated marker genes in normal peripheral blood. In this study, we have combined two new technologies in an effort to develop a more sensitive and reliable molecular diagnostic assay.

The first technology, real-time RT-PCR, is based on an on-line fluorescence detection system that is used to precisely quantitate gene expression levels (26). This high-throughput

assay allows for the sensitive detection and quantitation of gene expression and is quickly gaining recognition as the technology of choice for the precise measurement of gene expression levels (27). Real-time RT-PCR provides several advantages over conventional RT-PCR. First, the on-line fluorescence detection system is more sensitive than conventional RT-PCR, an important consideration, given the low concentration of circulating breast cancer cells in the peripheral blood. Second, real-time RT-PCR is quantitative, allowing the incorporation of genetic markers that are not truly breast cancer specific into the marker panel. The precise quantitation of real-time RT-PCR helps to differentiate background gene expression in the peripheral blood from gene overexpression associated with breast cancer cells.

The second technology involves the ability to enrich for malignant cells using novel density gradient centrifugation. Conceptually, enrichment technologies can improve sensitivity and decrease background gene expression. Prior studies have focused on magnetic cell capture, and there are a number of studies in the literature that suggest that positive selection of malignant epithelial cells using the BerEP4 monoclonal antibody can significantly enhance the ability to detect malignant epithelial cells in the peripheral blood with either ICC or RT-PCR (25, 28–32). The disadvantage of magnetic cell enrichment

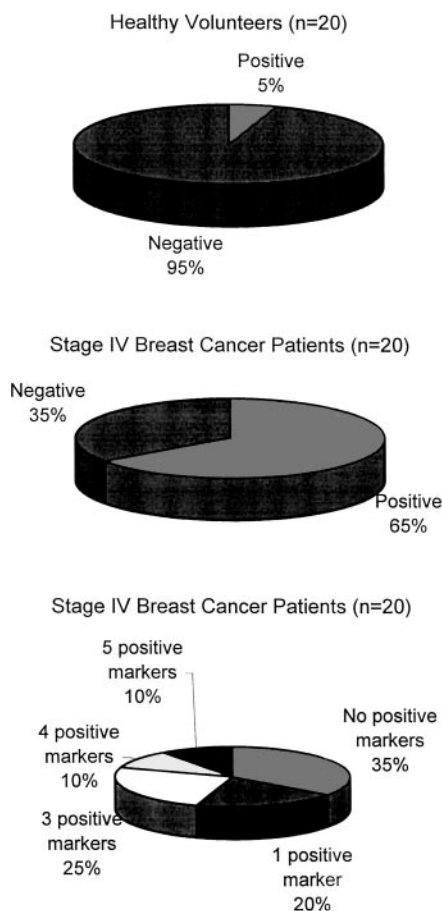


Fig. 5 Gene overexpression in stage IV breast cancer patients. The results of the pilot study are summarized in the pie charts. Gene overexpression thresholds were determined as indicated in "Materials and Methods." If gene overexpression was present for any individual marker, the specimen was considered to be positive.

is that it is both labor intensive and costly. Furthermore, it may not be practical to perform on peripheral blood specimens if transport to a centralized laboratory facility is required. Porous barrier density gradient centrifugation is designed to separate malignant cells from RBCs and hematopoietic cells based on the buoyant density of malignant cells using a two-compartment chamber and a porous barrier (15). Centrifugation protocols based on this technique are straightforward and permit preprocessing at the site of specimen acquisition. This study suggests that sophisticated density gradient separation techniques may prove to be more practical than magnetic cell enrichment with a similar capacity for enrichment of malignant cells.

The combination of porous barrier density gradient centrifugation and real-time RT-PCR appears to be extremely effective. Porous barrier density gradient centrifugation allows a 300-fold enrichment of breast cancer cells from peripheral blood (Fig. 1 and Table 1). Furthermore, this enrichment effectively eliminates the molecular background commonly seen with genes such as *mucl* and *CK19* (Fig. 2). Combining this enrichment protocol with real-time RT-PCR allows detection of 1 breast cancer cell in 15 ml of peripheral blood, or approximately

1 cell in 7.5×10^7 cells (Fig. 3). This represents a significant advance over current levels of sensitivity reported in the literature. This technology also lends itself readily to sampling of a larger volume of peripheral blood. In this study, 15 ml of blood were sampled, but it is likely that minor alterations of the density gradient centrifugation protocol could permit 50–100 ml of blood to be sampled easily, with a concomitant increase in sensitivity. In preliminary experiments, we have shown that increasing the blood volume results in a consistently stronger signal at very low concentrations of circulating breast cancer cells (data not shown).

Our clinical translation of these technologies confirms their potential. In our pilot study, 65% of stage IV breast cancer patients and 100% of stage IV breast cancer patients naïve to treatment had evidence of breast cancer-associated gene overexpression. These strong correlative data suggest that this molecular diagnostic assay needs to be studied in larger patient cohorts to precisely define the clinical relevance. Given the high percentage of stage IV patients with evidence of gene overexpression, it is easy to imagine that this combination of technologies could be used to monitor patients' response to treatment. Ultimately, further refinement is likely to allow for breast cancer screening and staging of patients with less advanced disease.

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