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13. ABSTRACT ***(Maximum 200 Words)***

A variety of genetic aberrations, including loss of heterozygosity (LOH), have been identified in primary breast cancers and their metastasis and shown prognostic significance. However, assessing these genetic events in tumor tissue provides information that is static, limited to the time the biopsy was obtained, and may not reflect those genetic events that continue to occur during disease progression and may have additional diagnostic and prognostic utility. Furthermore, serial tumor biopsy is not clinically practical or logistically feasible during patient treatment and follow-up. Because blood is a major route of metastatic dissemination, detection of circulating tumor-associated genetic markers may provide a less invasive and more informative method to assess disease. During the past year, we have demonstrated the presence of DNA microsatellites with LOH in the serum and bone marrow aspirates of early stage breast cancer patients and found a significant concordance with those present in the primary tumor. In addition, the detection of serum LOH was associated with those primary tumors having increased proliferation indices, and serum LOH incidence and frequency were correlated with advancing stage of disease. The findings may provide an innovative approach for surveillance, staging and the assessment of subclinical disease progression and response to therapy.

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TABLE OF CONTENTS

INTRODUCTION

Breast cancer initiation and progression involves the serial accumulation of a variety of genetic (DNA) alterations to the cell (1). One such event involves loss of heterozygosity (LOH) of DNA which has been shown to occur frequently in primary breast cancers and more so in metastatic disease (2). It has been suggested that these allelic losses associated with malignancy may involve potential tumor suppressor genes and/or metastasis regulator genes (3,4). Detection of these circulating tumor-associated DNA markers in the blood from cancer patients may serve as surrogate markers of subclinical disease progression and permit identification of high-risk patients early in their disease course (4-6). Furthermore DNA based genetic testing of serial blood samples offers an easily accessible route by which to evaluate ongoing genetic events which may have prognostic significance (7). This new approach provides for a minimally invasive method to monitor subclinical disease progression and offers a novel technique for potentially assessing response to therapeutic interventions in early stage breast cancer patients (8).

We have successfully demonstrated the detection of circulating DNA microsatellites for LOH in the serum of breast cancer patients (9). Furthermore the correlation of these findings with similar profiles in breast tumor tissues confirms the origin of these tumor-specific DNA fragments. Additionally, we have shown the frequencies of these DNA markers to increase with advancing tumor stage and ongoing investigations are evaluating correlations with other known clinical and pathologic prognostic factors associated with breast cancer. Because breast cancer is often diagnosed early and is associated with a prolonged time to recurrence we are in the process of following these patients with serial blood draws and marker analysis for long-term clinical correlations. We are currently developing a methodology to allow for high throughput and multiplexing of DNA markers in order to more efficiently and accurately evaluate multiple samples and establish a molecular marker profile for each individual patient.

Finally we have recently identified tumor-associated microsatellite alterations in the bone marrow of early stage breast cancer patients. These findings may prove critical as the most frequent site of distant recurrence in breast cancer is the bone and early detection and classification of tumor cells in bone marrow may better select patients for specific systemic therapies that target these tumor cells. Similar to our aims with serum markers of malignancy we are investigating these findings in a larger cohort of patients and with clinical follow-up to determine the prognostic and clinical implications of these findings.

With earlier disease detection, an increasing need exists to establish molecular methods for assessing occult disease progression, monitoring response to therapy, and ongoing genetic events associated with tumor biology, which will lead to improvements in the care of breast cancer patients

BODY

Aim Ia: Assessment of microsatellite markers for LOH in serum.

In this aim, we assessed breast cancer patients' serum for LOH using a panel of eight polymorphic microsatellite markers (TP53, D16S421, D17S855, D17S849, D14S5, D14S62, D10S197 and D8S321) on five chromosomes known to demonstrate frequent LOH in primary breast tumors. We have completed the assessment of serum collected from 71 breast cancer patients from the following stages of disease: AJCC stage I, 30 patients; AJCC stage II, 26 patients; AJCC stage III, 7 patients; and AJCC stage IV, 8 patients. DNA was isolated, purified and quantified from serum obtained pre-operatively from AJCC stage I-III patients and at the time of diagnosis from AJCC stage IV patients (**Appendix 1 and 2**). In addition, paired lymphocyte DNA was isolated from each patient to serve as a normal control. Following PCR, fragments were separated by gel electrophoresis and LOH was assessed using a fluorescent scanner and ClaritySC 3.0 software (Media Cybergenetics, Silver Spring, MD). The presence of LOH was scored if at least a 50% reduction in signal intensity was noted in one allele from serum DNA when compared to its corresponding allele in paired lymphocyte DNA (**Figure 1**). Samples were run in triplicate. Serum was collected from 30 healthy female donors and assessed in a similar manner.

We identified 20 (28%) of 71 patients' serum samples to demonstrate LOH for at least one of the microsatellite markers assessed. LOH on chromosome 17 was most common, occurring in 7 of 63 patients (11%) (**Table 1**).

Figure 1.

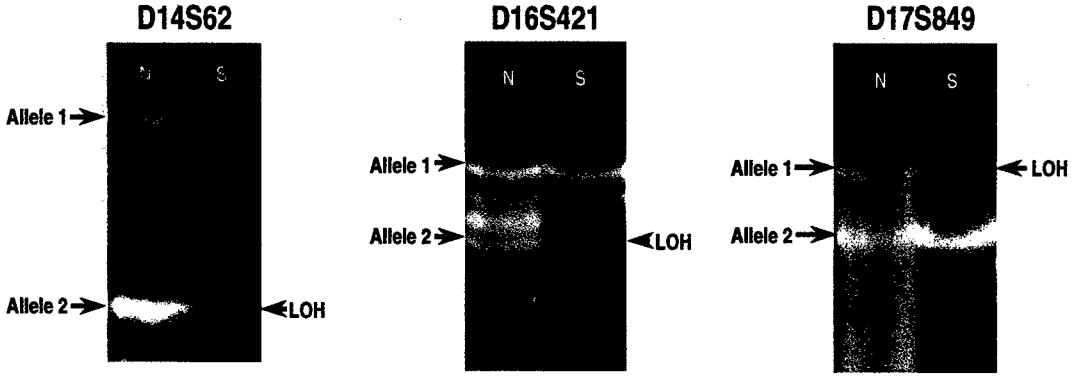


Table 1. LOH Frequency in Breast Cancer Patients' Serum Samples

Microsatellite Marker	LOH Frequency*
D17S849	11%
D16S421	10%
D10S197	7%
D8S321	6%
D14S62	1%
D17S855	1%
TP53	1%
D14S51	0%

*Positive LOH/total informative

Interestingly we found the frequency of LOH detected increased with advancing patient stage: 17% (5 of 30) stage I; 27% (7 of 26) stage II; 43% (3 of 7) stage III; and 63% (5 of 8) stage IV (**Table 2**).

Table 2. LOH Frequency Relative to AJCC Stage

AJCC Stage	LOH in Informative Patients*
I	5/30 (17%)
II	7/26 (27%)
III	3/7 (43%)
IV	5/8 (63%)

LOH was not detected in serum from any of the healthy female donors. Preliminary analysis of DNA quantitation using the Picogreen assay of early stage breast cancer patients' serum did not find an association between circulating DNA concentration and serum LOH markers. In addition, we have expanded our panel of markers for LOH detection to include other "hotspot" regions from chromosome arms 1p, 2p, 6q, 8q, 11p and 22q while dropping marker TP53 on chromosome 17 for its low frequency.

Aim Ib. Assessment of LOH markers in fluid of bone marrow aspirations.

For this aim because bone is such a common site of breast cancer recurrence, we sought to determine whether microsatellite markers associated with breast cancer could be detected in BM aspirates from patients with early stage breast cancer. Cell-free plasma from BM aspirates in 48 patients was collected intra-operatively from patients undergoing surgery for their primary breast cancer diagnosis (**Appendix 3**). This was a retrospective study of BM aspirates previously collected. Eight polymorphic microsatellite markers which correspond to regions that have been shown to demonstrate significant LOH suggesting sites of putative tumor suppressor and/or metastasis related genes were selected: D1S228 at *1p36;* D8S321 at *8qter-8q24.13;* D10S197 at *10p12;* D14S51 at *14q32.1-14q32.2;* D14S62 at *14q32;* D16S421 at *16q22.1;* D17S849 at *17pter-17qter* and D17S855 at *17q*. Methods were developed to optimize extraction of DNA from BM plasma. DNA was isolated from acellular fluid obtained from BM aspirations, purified and quantified. Paired lymphocytes were collected from each patient and DNA extracted to serve as normal control for each PCR reaction. Following PCR, fragments were separated by gel electrophoresis and LOH was assessed using a fluorescent scanner and ClaritySC 3.0 software. LOH was scored in a manner similar to serum DNA analysis. LOH was identified in 11 (23%) of 48 patients' BM aspirates. LOH was most commonly identified at microsatellite marker D14S62 occurring in 4 (12%) of 34 informative patients. Microsatellite markers demonstrating LOH at D1S228 and D14S51 occurred in 3 (8%) of 38 informative patients each, followed by LOH at D8S321 (5%), D10S197 (4%), and D17S855 (3%). No LOH was detected for microsatellite markers D16S421 and D17S849 (**Table 3**).

Table 3. LOH frequency in Breast Cancer Patients' Bone Marrow Aspirates

Microsatellite marker	LOH in BM aspirates/informative cases (%)
D14S62	4/34 (12%)
D14S51	3/38 (8%)
D1S228	3/38 (8%)
D8S321	2/39 (5%)
D10S197	1/26 (4%)
D17S855	1/37 (3%)
D17S849	0/31 (0%)
D16S421	0/28 (0%)

No LOH was detected for any of the microsatellite markers assessed BM aspirates collected from five healthy female donors. There was an increased association between the presence of LOH in the BM and advanced disease stage. Six (19%) of 32 AJCC stage I patients demonstrated LOH for at least one marker, in contrast to 4 (31%) of 13 AJCC stage II patients, and 1 (50%) of 2 AJCC stage III patients (**Table 4**). In addition, circulating microsatellites for LOH detected in early stage disease patients was associated with primary tumors demonstrating increased proliferative indices as evaluated by Ki67, S phase, and ploidy status (**Appendix 4**).

Table 4. Association of LOH in patients' BM aspirates with AJCC stage

AJCC Stage	Patients with LOH in BM/ total Patients (%)
I	6/32 (19%)
II	4/13 (31%)
III	1/2 (50%)

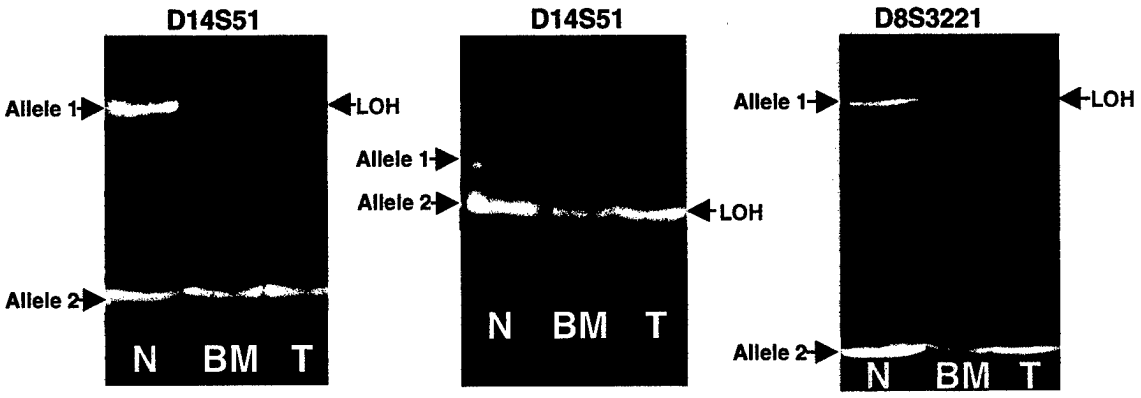
This study demonstrates the novel finding of tumor-related genetic markers in BM aspirate plasma of early stage breast cancer patients and provides a unique approach for assessing subclinical systemic disease progression, and the monitoring of breast cancer patients.

Aim Ic. Correlation of LOH microsatellite markers in paired bone marrow and primary tumors.

To determine whether a correlation existed between LOH detected in early stage breast cancer patients' bone marrow and primary tumors, we assessed 10 match-paired specimens with identical microsatellite markers. DNA was isolated from paired primary tumor and bone marrow specimens analyzed using 8 microsatellite markers (D1S228, D8S321, D10S197, D14S51, D14S62, D16S421, D17S849 and D17S855). Gel electrophoresis was performed and results assessed for each specimen using a positive cut-off for LOH of ≥50%. Among all ten samples, LOH detected in the bone was also present respectively in the primary tumors (**Figure 2**). In this pilot study, no LOH was identified in the bone marrow that was not found in the patients paired primary tumors. We are currently investigating a larger cohort of patients with additional microsatellite markers to determine if these early findings remain consistent for all disease stages and among the different length microsatellite repeats.

In addition, we are in the process of assessing microsatellite markers in match-paired in serum and primary tumors from the same patient.

Figure 2.



Aim IIa. Assessment of circulating tumor cells in blood by qualitative RT-PCR and correlation to LOH in serum

In this study we have assessed blood from 65 breast cancer patients (average age: 56 years; range: 29-83 years) diagnosed with the following AJCC stage: stage I, 26 patients; stage II, 23 patients; stage III, 9 patients; and stage IV, 7 patients (Appendix 5). RNA was extracted from cells in the blood and analyzed using an ultrasensitive electrochemiluminescence detection solution phase technology (IGEN) with specific probes for the presence of four tumor markers (β-HCG, c-MET, GalNAc and MAGE-3) previously demonstrated to be expressed in breast cancer specimens. The most frequently expressed marker detected was GalNAc, 24 patients (37%); followed by β-HCG, 22 patients (34%); MAGE-3, 9 patients (14%); and c-MET, 7 patients (11%). In 32/65 (49%) patients blood samples demonstrated the presence of only one marker. The addition of multiple markers increased the number of patients with a positive blood sample to 45 (69%) from 32, improving sensitivity by 20%. No markers were detected in the blood from any of the forty healthy donor volunteers. Correlation of marker expression will be performed with known clinicopathologic prognostic parameters associated with breast cancer. Furthermore, we are currently in the process of collecting and assessing and assessing matched-paired serum samples for LOH and circulating tumor cells in the blood by qualitative and quantitative RT-PCR for correlation.

Aim IIb. Assessment of circulating tumor cells in blood by quantitative RT-PCR

Currently we are developing and optimizing markers used in IIa for quantitative RealTime RT-PCR (qRT). Primers, probes and standards have been developed. The assays for individual markers are being evaluated on controls for specificity and sensitivity. The optimized assays will be used for assessment of blood from patients.

Aim IIc. Assessment of isolated tumor cells in blood

Currently, we are assessing immunostaining of apoptotic cells in culture and tissue sections. The objective is develop an optimized assay that can be informative and reliable. We find single cells are difficult to assess for apoptosis whereby, clumps of cells are easier to assess. Currently we are developing techniques to improve isolation of tumor cells from blood. Different types of magnetic beads and antibodies are being tested to get optimal cell isolation.

Aim IIIa. Neoadjuvant treatment analysis

To date we have not analyzed patients receiving neoadjuvant treatment.

KEY RESEARCH ACCOMPLISHMENTS

- Developed a panel of informative microsatellite markers for LOH assessment in breast cancer
- Developed a method to qualitatively and quantitatively assess the presence of circulating tumor DNA in the blood breast cancer patients
- Developed a method to qualitatively and quantitatively assess the presence of circulating tumor DNA in the bone marrow of breast cancer patients
- Demonstrated the presence of circulating DNA with LOH in the serum of breast cancer patients
- Demonstrated the presence of circulating DNA with LOH in the acellular plasma from bone marrow aspirations of breast cancer patients
- Determined that the presence of circulating microsatellite markers for LOH in breast cancer patients' serum correlates with those present in the primary tumor
- Determined that the presence of circulating microsatellite markers for LOH in breast cancer patients' bone marrow aspirates correlate with those present in the primary tumor
- Demonstrated that LOH in early stage breast cancer patients' serum is associated with primary tumors having increased mitotic activity and proliferative indices
- Demonstrated that serum LOH incidence and frequency is associated with advancing patient stage
- Demonstrated that bone marrow LOH is associated with increasing primary tumor size and infiltrating lobular type histology
- Demonstrated that bone marrow LOH presence is associated with advancing patient stage
- Demonstrated detection of circulating tumor cells in breast cancer patients using molecular markers, c-Met, U-MAGE, and β-hCG

REPORTABLE OUTCOMES

A. Manuscripts

1. Taback B, Shu S, Giuliano AE, Hansen NM, Singer FR, Hoon D. Detection of Tumor-Specific Genetic Alterations in Bone Marrow from Early Stage Breast Cancer Patients (submitted)
2. Taback B, Chan AD, Kuo CT, Bostick P, Wang H, Giuliano AE, Hoon D. Detection of Occult Metastatic Breast Cancer Cells in Blood by a Multi-molecular Marker Assay: Correlation with Clinical Stage of Disease. Cancer Res, 61: 8845-8850, 2001
3. Taback B, Giuliano A, Hansen N, Hoon D. Microsatellite Alterations Detected in the Serum of Early Stage Breast Cancer Patients. *In:* P. Anker and M. Stroun (eds.), Circulating Nucleic Acids in Plasma or Serum II. Ann NY Acad Sci 945: 22-30, 2001

B. Abstracts

1. Taback B, Shu S, Hansen N, Giuliano A, Hoon D. Detection of Tumor-Specific DNA in Breast Cancer Patients' Sera. Department of Defense Era of Hope Proceedings, Vol II: P24-7, 2002
2. Taback B, Giuliano AE, Hansen N, Hoon D. Circulating DNA Microsatellites with LOH in Early Stage Breast Cancer Patients Serum is Associated with Increased Tumor Proliferation. Clinical Chemistry, 47: 365, 2001
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4. Taback B, Giuliano AE, Nguyen D, Nakayama T, Hansen N, Martino S, Fournier PJ, Hoon D. Tumor-Related Free DNA Microsatellites Detected in Breast Cancer Patients Serum Correlates with Disease Progression. Proceedings of the American Society of Clinical Oncology19: 606a, 2000

CONCLUSIONS

During the past year we developed and standardized techniques for the detection and assessment of circulating tumor-associated DNA in the serum and bone marrow aspirates from patients with breast cancer. Our studies demonstrate that tumor-specific DNA for LOH can be identified in the blood of breast cancer patients. In addition, for the first time, we have demonstrated this occurrence in the bone marrow of early stage breast cancer patients. These findings are significant because blood is a significant route for metastasis and the most frequent site of systemic recurrence is bone. Furthermore, because ongoing genetic events have been associated with cancer progression and may provide valuable diagnostic and prognostic information, detection of these alterations in the blood and bone marrow may provide a novel approach for improving patient staging, identification of high risk patients for additional therapies earlier in their disease course, assessing subclinical response to therapy, and occult disease surveillance.

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APPENDICES

1. Taback B, Shu S, Hansen N, Giuliano A, Hoon D. Detection of tumor-specific DNA in breast cancer patients' sera. DoD Era Hope Proc II:24-27, 2002.
2. Taback B, Giuliano A, Hansen N, Hoon D. Microsatellite alterations detected in the serum of early stage breast cancer patients. In: Anker P, Stroun M, editors. Circulating Nucleic Acids in Plasma or Serum II. Ann N Y Acad Sci 945:22-30, 2001.
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5. Taback B, Chan A, Kuo C, Bostick P, Wang H, Giuliano A, Hoon D. Detection of occult metastatic breast cancer cells in nlood by a multimolecular marker assay: correlation with clinical stage of disease. Cancer Res 61:8845-8850, 2001.

DETECTION OF TUMOR-SPECIFIC DNA IN BREAST CANCER PATIENTS' SERA

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The benefit of early detection of breast cancers is well established. Advances in screening technologies have resulted in a dramatic shift towards diagnosing smaller tumors with improved prognosis. Additionally, lymphatic mapping and sentinel node biopsy has permitted for a more comprehensive evaluation for occult metastasis, which may upstage patients' disease. However, it has been suggested that lymph node metastasis may merely serve as a surrogate marker for systemic disease since hematogenous dissemination can occur in patients without evidence of lymph node involvement. More so, lymph node evaluation can not be performed serially to monitor disease progression. Therefore methods that can identify early systemic progression directly through this blood-borne route may provide a valuable aid to identifying and monitoring patients at increased risk for relapse. We propose molecular detection of tumor-specific circulating DNA in the blood of breast cancer patients may demonstrate utility as a surveillance tool. Blood was collected preoperatively from 63 patients diagnosed with breast cancer (30 patients AJCC stage I, 26 stage II and 7 stage III) and 8 patients during diagnosis of stage IV disease. Serum from each of these patients as well as from 30 healthy female donors was assessed for loss of heterozygosity (LOH) using 8 microsatellite markers on 5 chromosomes (8q, 10p, 14q, 16q, 17q) that frequently demonstrate LOH in primary breast cancers. DNA was isolated and quantitated from 1 ml of acellular serum. Analysis of LOH was performed by gel electrophoresis using a fluorescent scanner. Overall, 28% (20 of 71) of the patients had LOH present in their serum. The incidence of LOH increased with advancing stage: 17% (5 of 30) stage I; 27% (7 of 26) stage II; 43% (3 of 7) stage III; and 63% (5 of 8) stage IV. LOH on chromosome 17 was most common occurring in 7 of 63 patients (11%). LOH was not detected in serum from any of the healthy female donors.

These results reveal the presence of tumor-specific DNA in the blood of breast cancer patients and reflect a correlation to disease stage. This study offers an innovative approach to aid in the assessment of disease progression using molecular analysis of cancer patients' blood.

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Microsatellite Alterations Detected in the Serum of Early Stage Breast Cancer Patients

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ABSTRACT: **Breast cancer is the most common malignancy affecting women. Advances in screening have resulted in an increasing trend towards detecting earlier stage tumors associated with a longer disease-free survival. Because of this prolonged latency period, it is critical to identify patients early in their disease course who are at increased risk for recurrence, whereby treatment decisions may be altered accordingly based on more precise information. Molecular markers that demonstrate prognostic importance as well as utility for assessing subclinical disease progression offer one such approach. Specifically, circulating microsatellite alterations that reflect those genetic events occurring in tumors and that can be serially assessed through a minimally invasive procedure are a logistically practical method. In this study, serum was collected preoperatively from 56 patients with early stage breast cancer (AJCC stages I/II) and assessed for loss of heterozygosity (LOH) using 8 microsatellite markers. Twelve (21%) of 56 patients demonstrated LOH in their serum for at least one marker. Histopathologic correlation revealed an association between the presence of circulating LOH in serum and those tumors with increased proliferation indices as characterized by an increased diploid index, elevated MIB-1 fraction, and abnormal ploidy. These findings demonstrate the presence of circulating microsatellite alterations in the serum from patients with early stage breast cancer. The association of known poor prognostic features found in tumors with increased nuclear activity not only suggests a possible etiology for their presence, but also offers a potential blood-based surrogate marker for this disease that may demonstrate clinical utility in long-term follow-up studies.**

KEYWORDS: **Breast cancer; Microsatellite alterations; Loss of heterozygosity (LOH); Serum**

INTRODUCTION

Breast cancer remains a major public health concern in the United States. It is the most frequent anatomical site of cancer diagnosis among American women, with an estimated 190,000 new cases for 2001.[1] More so, with 40,000 projected breast cancer–related deaths this year, it continues to be the most common cause of cancer mortality among women aged 20 to 59 years and is second only to lung cancer in

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women 60 years and older.[1] Advances in breast cancer screening and an enhanced public awareness have resulted in a dramatic increase in the detection of early stage breast cancer that is associated with a more favorable prognosis, as compared to those cases in which the disease is more advanced.[2] It is no wonder that there is considerable interest not only in the early detection, but also in the accurate staging of patients, and thereby in the identification of those who may be at increased risk for recurrence.

Presently, the histologic status of the axillary lymph nodes remains the single most important prognostic factor for predicting overall survival in patients diagnosed with breast cancer.[3,4] However, with the increasing trend towards early breast cancer detection, many studies have confirmed that almost two-thirds of patients undergoing breast cancer surgery for invasive disease will have negative axillary lymph nodes by histopathologic assessment.[5–8] Sentinel lymph node surgery, a less invasive procedure, in this instance has shown equivalent accuracy for predicting the pathologic status of the axilla and therefore may spare a majority of patients from conventional axillary dissection and its potential for increased morbidity.[9–12] In addition, the sentinel node technique allows for a more thorough, efficient, and cost-effective search for the presence of occult lymph nodes.[13] Unfortunately, 20–30% of node-negative patients will develop recurrent disease and many investigators have sought to identify additional primary tumor characteristics that would provide equivalent, if not supplementary, prognostic information.[14–20] Factors that have limited these attempts include the following: (1) tumors are composed of a heterogenous collection of cells of which the metastatic clone may only represent a subpopulation of the primary tumor and thus may not be accurately identified when assessing the tumor in total; (2) evaluating a primary tumor only provides information at one point of the tumor history and may not account for those individual variations that occur in response to a particular treatment; and (3) because of the relatively indolent nature of most breast cancers, assessing the primary tumor does not provide an accurate method to serially assess those ongoing genetic events that can occur during disease progression that may be of prognostic utility. Finally, because breast cancers spread through hematogenous as well as lymphatic routes, the additional assessment of this alternative source for metastatic progression provides a logical and easily accessible approach to augment current staging procedures and identify potential prognostic markers of disease progression and response to treatment.

The advent of the polymerase chain reaction (PCR) to amplify nucleic acid sequences to a sufficient copy number for detection has afforded investigators the opportunity to identify occult tumor cells in the blood and other organ sites with increased sensitivity over standard radiographic and light microscopic imaging techniques.[21–27] These methods can identify a single malignant cell among a background of 1×10^5 normal cells and may identify patients at increased risk for recurrence.[28,29] However, limitations in sensitivity and specificity have been shown to exist with this technique, including tumor cell heterogeneity for mRNA marker expression, coincidental expression of the specific mRNA marker by normal tissue, and the inherent lack of stability associated with mRNA that may impair assay sensitivity.[30] More recently, investigators have evaluated genetic alterations in tumor cells that can be identified in blood that may act as markers for disease.[31–34] These microsatellite alterations, such as the loss of heterozygosity (LOH), are unique to

tumor cells and have been shown to demonstrate clonality. Therefore, they are highly specific markers that can be used to assess disease presence.[35,36]

The presence of circulating nucleic acids in plasma/serum was first described by Mandel and Métais in 1947.[37] It was not until 30 years later that circulating free DNA was demonstrated to be elevated in patients with cancer. In 1977, Leon *et al.*,[38] using a radioimmunoassay for DNA quantitation, found free DNA levels to be markedly elevated in cancer patients when compared to normal controls (mean concentration: 13 ng/mL vs. 180 ng/mL, respectively). Furthermore, serum DNA levels were twice as high in patients with metastatic disease as compared to patients without metastasis, thus inferring that circulating free DNA may be used to monitor disease progression. However, it was not until 1982 when Stroun, Anker, and coworkers characterized the nature of this circulating DNA in cancer patients as from tumor cell origin (i.e., as opposed from other sources such as activated lymphocytes) that the field of using genetic-based markers in blood as surrogates of disease was born.[39,40]

The historical methods for DNA extraction, quantitation, and assessment were time-consuming, lacked specificity, and were associated with exposure to potentially hazardous radioactive and toxic organic solvents.[41,42] With current commercially available kits, quality DNA can be rapidly and efficiently extracted from serum and plasma in a limited amount of time. Furthermore, newer blood collection tubes such as the CORVAC serum separator tube (Sherwood-Davis & Geck, St. Louis, MO) ensure a more reliable transport by displacing a physical gel barrier, after centrifugation, between the two compartments (clotted blood and serum) to prevent ongoing contamination of the serum from DNA that may be released as cells undergo lysis.

To date, despite the widespread prevalence of breast cancer, little is known of its association with free DNA in the serum. Studies in other tumor models have demonstrated that detection of circulating microsatellite alterations in plasma/serum of cancer patients is frequent and therefore may have clinical utility as a diagnostic and/or prognostic tool. The purpose of this study was to demonstrate the presence and to evaluate the frequency of genetic alterations, particularly LOH, in circulating DNA among American women with breast cancer. Eight microsatellites that map to regions previously shown to demonstrate frequent LOH in primary breast tumors on five chromosomes, namely, 8, 10, 14, 16, and 17, were evaluated.

MATERIALS AND METHODS

Fifty-six patients with a diagnosis of breast cancer at the John Wayne Cancer Institute/Saint John's Health Center were evaluated. Blood was collected preoperatively from 30 stage I and 26 stage II breast cancer patients undergoing breast-conserving surgery and lymphatic mapping with sentinel lymph node biopsy. In addition, blood was taken from 30 healthy female donors. Serum separator tubes were used to collect all blood samples. Following centrifugation, serum was isolated and DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Lymphocytes were collected from each patient and DNA was extracted using DNAzol (Molecular Research Center, Cincinnati, OH) for comparison. PCR was performed on each patient's paired samples (normal lymphocyte DNA and serum DNA) using FAM-labeled primer sets for 8 microsatellite markers: TP53, D17S855,

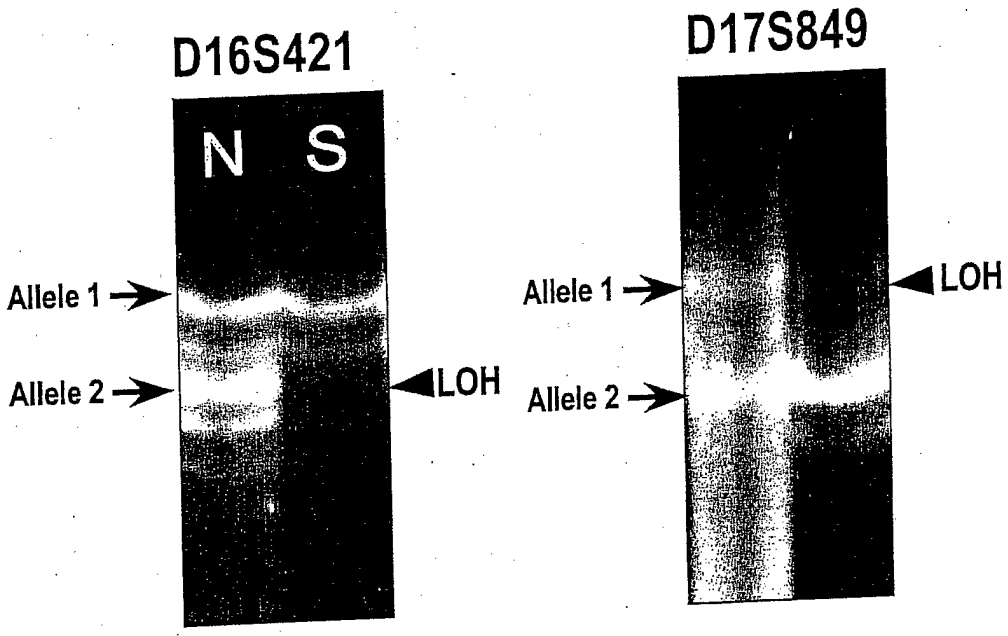

FIGURE 1. Representative images demonstrating LOH in breast cancer patients' serum at D16S421 and D17S849. Analysis for patient-paired specimens: N, lymphocytes; S, serum. Arrows represent the position of the alleles.

D17S849, D16S421, D14S62, D14S51, D10S197, and D8S321 (Research Genetics, Huntsville, AL).

PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel (PAGE) and the images were scanned using a GenomyxSC fluorescent optical scanner (Beckman Coulter, Fullerton, CA). Densitometric analysis was performed with the ClaritySC 3.0 software (Media Cybergenetics, Silver Spring, MD) and LOH was scored if a greater than 50% reduction in signal intensity occurred in one allele when compared to the respective allele in the corresponding lymphocyte DNA.

RESULTS

Twelve of 56 patients (21%) demonstrated LOH, in preoperatively collected serum samples, for at least one of the microsatellite markers assessed (FIG. 1). Nine patients (16%) exhibited LOH for only one of the molecular markers and 3 patients (5%) had LOH on two markers. For the majority of patients (79%) with early stage breast cancer, no LOH was detected in the serum for any of these circulating microsatellites. For informative cases, the most frequent microsatellite marker demonstrating LOH was D16S421, occurring in 4 of 37 patients (11%). This was followed by D10S197 demonstrating LOH in 9% of patients and D8S321 in 8% of patients (TABLE 1). As expected, there was an increase in the presence of circulating microsatellites for LOH in patients with AJCC stage II disease versus stage I (27% vs. 17%, respectively; TABLE 2). Further, we evaluated the 12 patients demonstrating LOH in their serum for clinical correlation with known histopathologic factors

TABLE 1. Frequency of LOH in breast cancer patients' serum samples

Microsatellite marker	LOH frequency[a]
D16S421	11%
D10S197	9%
D8S321	8%
D17S849	7%
D14S62	2%
D17S855	0%
D14S51	0%
TP53	0%
Total	21%

[a]Positive LOH/total informative patients.

TABLE 2. Detection of LOH according to AJCC stage

AJCC stage	LOH in total patients
I	5/30 (17%)
II	7/26 (27%)

associated with a poorer prognosis: tumor size, grade, angiolymphatic invasion, Bloom-Richardson score, ploidy, DNA index, S phase fraction, MIB-1, HER2, and p53 protein overexpression. In all but two cases, circulating LOH was associated with primary tumors demonstrating abnormal ploidy, increased diploid index, and MIB-1. Serum from 30 healthy female donors did not demonstrate LOH for any of the microsatellite markers assessed. Finally, serum from 5 patients identified as having only DCIS was evaluated for LOH. No circulating LOH was detected in any of these patients or those with invasive tumors less than 1 cm in size.

DISCUSSION

This is the first comprehensive study to demonstrate the presence of circulating DNA microsatellite alterations in the serum from a significant cohort of early stage breast cancer patients. The current study provides substantial evidence that tumor-associated DNA microsatellites are present in the serum from patients with early invasive breast cancer and therefore may serve as surrogate markers of disease. These findings are of significant clinical importance as most patients with early stage breast cancer (AJCC stages I and II) can be adequately staged and treated with contemporary breast conservation surgery, lymphatic mapping/sentinel lymph node biopsy, adjuvant radiation, and chemotherapy. However, 20–30% of patients with negative lymph nodes will develop a recurrence and, for that reason, additional

methods are needed to identify and follow this high-risk group of patients. DNA-based molecular testing through blood surveillance offers an innovative and practical approach. We found an overall incidence of serum LOH of 21% in patients with early stage breast cancer. LOH was more frequent in patients with advanced disease: 17% for AJCC stage I versus 27% for AJCC stage II. Furthermore, we found that the presence of circulating LOH in early stage breast cancer patients was associated with primary tumors of increased mitotic activity.

Previous studies demonstrating the presence of circulating microsatellite alterations for LOH have identified these findings in the *plasma* of breast cancer patients.[43–46] Reported incidences of plasma LOH have ranged from 27% to 66%. However, a very heterogeneous population of tumors were evaluated for each study, with all tending toward larger tumor sizes and more advanced stages of disease. During our preliminary investigations, we evaluated paired plasma and serum from patients and found greater yields of free DNA in serum using the Picogreen assay (Molecular Probes, Eugene, OR). In addition, most studies demonstrate no correlation with any clinical or pathologic parameters.[43–45] More clinically pertinent would be to identify circulating molecular markers early in the disease course that may have prognostic significance and could serve as surrogates for monitoring tumor progression and/or response to treatment.

Despite the worldwide frequency of this disease, there is a paucity of reports in the literature identifying circulating nucleic acids in the plasma/serum of breast cancer patients. More so, many have reported difficulty with at least some aspect of this technique.[44,45] Chen *et al.* demonstrated that they could improve detection of circulating LOH by assessing serum instead of plasma and by the addition of laser fluorescence and an automatic sequencer in place of radioactive autoradiography (48% vs. 21%, respectively).[44] Although they report a higher detection rate, all of their tumors were 1 cm or greater in size and more than half had lymph node involvement or unknown lymph node status, indicating a patient population with more advanced disease. This technique may prove more sensitive than conventional densitometric analysis of standard gels, but additional direct comparisons are required. In order to determine any clinical utility for this assay, larger scale studies are needed with well-defined patient populations.

Breast cancer is one of the most common diseases to affect women, with approximately one in eight being diagnosed during their lifetime. More disturbing is that about 15% will die of their disease despite improvements in radiographic screening methods, surgical staging techniques, and adjuvant chemotherapy. Thus, additional methods are needed that would further improve current staging techniques and that would identify patients at increased risk for recurrence who may benefit from more aggressive treatment up front. More so, methods that permit the monitoring of occult disease progression may distinguish patients who are not responding to current treatment regimens and/or identify patients at increased risk for relapse, altering decision-making treatment plans earlier in the disease course that may prove beneficial. Braun *et al.* demonstrated that the persistence of breast cancer micrometastasis in the bone marrow following adjuvant chemotherapy was associated with a worse prognosis.[47] Although promising, the feasibility of performing this invasive technique serially on patients to monitor disease may be limiting. To date, the most widely used method for disease surveillance is radiographic imaging. However, as a screening tool, this

method is costly, can be time-consuming to the patient, and more importantly requires the presence of a significant tumor burden for detection. Ideally, a blood test would provide a logistically practical approach for assessing disease progression through a minimally invasive route that is easily accessible. Furthermore, blood-based DNA detection assays are highly specific and, with PCR amplification, provide an extremely sensitive method to assess submicroscopic disease. If long-term studies prove these results to be clinically significant, these findings would afford the physician the opportunity to intervene earlier in the disease course, which may prove to be of substantial benefit to the patient. This has already been demonstrated by clinical studies showing a survival advantage with chemotherapy in node-negative breast cancer patients.[48,49] However, not all of these patients would have subsequently developed recurrent disease, and therefore a higher degree of discrimination is needed when exposing all patients to a costly and potentially toxic therapy.[50] The true benefit of adjuvant chemotherapy for early stage breast cancer will only occur in those patients who have metastatic disease that currently would elude present-day detection methods. Techniques that enhance the identification of early metastasis will provide a more selective and cost-effective use of adjuvant therapy. The detection and surveillance of circulating tumor-associated microsatellite alterations may provide a novel approach to the monitoring of breast cancer and thus provide valuable information that may aid treatment decisions.

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CIRCULATING DNA MICROSATELLITES WITH LOH IN EARLY STAGE BREAST CANCER PATIENTS SERUM IS ASSOCIATED WITH INCREASED TUMOR PROLIFERATION

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LOH of DNA microsatellites is a common genetic event occurring in primary breast tumors and can be detected in blood. However, clinicopathologic correlations are limited and inconsistent which may be due to the varied patient populations assessed and differences among techniques. The purpose of this study was two-fold: 1) demonstrate the presence of LOH in the serum from early-stage breast cancer patients (EBC), and 2) determine whether correlations exist with known histopathologic parameters.

Preoperative serum was obtained from 56 patients (stage I and II) undergoing segmental mastectomy and sentinel lymphadenectomy, and assessed for LOH with 8 microsatellite markers: TP53, D16S421, D17S855, D17S849, D8S321, D10S197, D14S62 and D14S51. Twelve of 56 (21%) patients demonstrated LOH for at least one marker, most frequent was D16S421 (11%). No LOH was detected in 5 patients with DCIS or those with tumors < 1 cm. In all, but two cases, circulating LOH was associated with primary tumors demonstrating abnormal ploidy, increased diploid index and MIB-1 fraction.

This comprehensive study provides evidence demonstrating the presence of free tumor-related genetic markers in EBC serum. The association of these findings with tumors manifesting known pathologic features consistent with increased proliferation suggests a possible etiology for their presence.

Detection of Tumor-Specific Genetic Alterations in Bone Marrow from Early Stage Breast Cancer Patients

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ABSTRACT

Detection of genetic markers associated with early breast cancer may prove clinically relevant for identifying patients at increased risk for relapse. Loss of heterozygosity (LOH), a specific genetic aberration, commonly occurs during breast cancer initiation and metastasis. Early detection of tumor metastasis to bone marrow (BM) using conventional histochemical techniques has been limited due to sub-optimal efficiency and sensitivity. Because bone is such a common site of breast cancer recurrence, we sought to determine whether microsatellite markers associated with breast cancer could be detected in BM aspirates from patients with early stage breast cancer. Cell-free plasma from BM aspirates in 48 patients was assessed for LOH using a panel of eight polymorphic microsatellite markers. LOH was detected in 11 (23%) of 48 patients' BM aspirates. Advancing AJCC stage was associated with an increased incidence of LOH. Concordance was present between LOH identified in BM aspirates and matched-pair primary tumors. No samples contained detectable tumor cells on routine histology. This study demonstrates the novel finding of tumor-related genetic markers in BM aspirate plasma of early stage breast cancer patients and provides a unique approach for assessing subclinical systemic disease progression, and the monitoring of breast cancer patients.

INTRODUCTION

Bone is the most frequent site of systemic spread of breast cancer (1, 2). Once metastases are clinically apparent overall prognosis is poor. Undetected occult tumor cells contribute to disease recurrence and therefore methods to identify subclinical disease (micrometastasis) may improve staging and guide additional therapeutic decisions. Historically, conventional cytologic assessment of blood and bone marrow (BM) aspirates has been performed with limited success (3, 4). Immunocytochemical techniques using antibodies specific to epithelial antigens have improved sensitivity and can identifying a single tumor cell amongst a background of >1 million normal cells (5, 6). Enrichment methods with antibody-magnetic bead conjugates of BM aspirates have demonstrated the presence of occult tumor cells in early stage breast cancer patients (5, 7). Recently it has been shown that detection of micrometastasis in the BM of early stage breast cancer patients is an independent prognostic risk factor (8, 9). However, accurate microscopic analysis of many cytologic samples requires considerable cytopathologic expertise and can be tedious particularly if performed serially to assess disease progression and or response to treatment. Additionally, the variable specificity of individual antibodies used to detect single cells has been questioned (8, 10, 11). Finally these assay systems cannot characterize the biologic behavior of the cells being detected and thus many may represent dormant tumor cells, apoptotic cells, nonpathologic tumor cells or displaced normal breast epithelial cells.

A variety of serial genetic changes have been implicated in the initiation and progression of solid tumors. One such event, allelic imbalance (loss of heterozygosity; LOH) has been shown to occur commonly in primary breast tumors and with additional

frequency in metastasis (12-15). Furthermore, there is emerging evidence to suggest that microsatellite markers for detecting LOH at specific chromosome loci may have important clinical prognostic correlations (12, 16, 17). However, examination of an excised primary tumor specimen may be of limited value in that it provides information of those genetic events that have occurred and not ongoing alterations which may be of clinical relevance, either prognostically or for therapeutic decisions. Additionally, because of the potentially long latent period that may exist between early breast cancer diagnosis and clinically detectable systemic recurrence, improved assessment methods are needed for serial surveillance of occult disease progression and monitoring response to therapy.

Recently it has been shown that free tumor-associated DNA can be identified in the serum and plasma from patients with melanoma, breast, lung, renal, gastrointestinal and head and neck tumors (18-32). Furthermore a high-quality concordance has been shown to exist between the genetic alterations (i.e.; LOH, microsatellite instability, mutations) found in circulating tumor DNA and those from the primary tumor suggesting a potential surrogate tumor marker (20-22, 27, 28). Early studies have shown prognostic importance of circulating microsatellite markers for LOH in blood (21, 24). Since BM is a common site for breast cancer recurrence, we sought to determine whether BM aspirate plasma could provide a viable source to detect tumor-specific DNA associated with systemic metastasis from early stage breast cancers.

MATERIALS AND METHODS

Surgical Specimens and DNA Isolation. BM aspirates were collected in 4.5 ml sodium citrate tubes (Becton Dickinson, Franklin Lakes, NJ) through bilateral anterior iliac approach from 48 consecutive patients as follows: ductal carcinoma in situ (DCIS), 1 patient; American Joint Committee on Cancer (AJCC) stage I, 32 patients; AJCC stage II, 13 patients; and AJCC stage III, 2 patients; undergoing surgical resection of their primary breast cancer at the Saint John's Health Center/ John Wayne Cancer Institute. In addition, five healthy female volunteer donors contributed BM aspirate samples for controls. All patients signed an informed consent form approved by the Institutional Review Board. Bone marrow was drawn and (cell-free supernatant) plasma was immediately separated by centrifugation (1000 x g, 15 min), filtered through a 13-mm serum filter (Fisher Scientific, Pittsburgh, PA) to remove any potential contaminating cells, aliquoted and cryopreserved at -30°C. For normal genomic DNA controls, whole blood was collected from each patient spotted and stored on FTA blood cards (Fitzco, Minneapolis, MN) prior to DNA isolation. DNA was extracted from one ml of BM aspirate plasma using QIAamp extraction kit (Qiagen, Valencia, CA) as previously described (23).

To assess the correlation of LOH found in the BM and that of the primary breast tumor, DNA was isolated from 10 μm sections cut from paraffin-embedded tissue blocks. Samples were deparaffinized, microdissected using laser capture microscopy (Arcturus, Mountain View, CA) from normal tissue and incubated with proteinase K in lysis buffer (50 mM Tris-HCl, 1 mM EDTA and 0.5% Tween 20) at 37° C overnight and then heated at 95° C for 10 min.

Additionally, each BM aspirate was assessed for the presence of occult tumor cells by conventional histologic staining methods using Hematoxylin and Eosin (H&E).

Microsatellite markers and PCR amplification. Eight polymorphic microsatellite markers which correspond to regions that have been shown to demonstrate significant LOH suggesting sites of putative tumor suppressor and/or metastasis related genes were selected: D1S228 at *1p36*; D8S321 at *8qter-8q24.13*; D10S197 at *10p12;* D14S51 at *14q32.1-14q32.2*; D14S62 at *14q32*; D16S421 at *16q22.1*; D17S849 at *17pter-17qter* and D17S855 at *17q*. All primer sets were obtained from Research Genetics (Hunstville, Al) and sense primers were labeled with a fluorescent dye: 5-(and-6)-carboxyfluoroscein, FAM. Approximately 20 ng of genomic DNA was amplified by PCR in 25 μl reactions containing 1X PCR buffer (Perkin Elmer, Foster City, CA), 6 pmol of each primer, 1 unit of Taq DNA polymerase, 2.5 μM deoxynucleotide triphosphates, and 1.5 mM $MgCl_2$. Forty PCR cycles were performed with each cycle consisting of 30 s at 94°C, 30 s at 50-56°C, and 90 s at 72°C, followed by a final extension step of 72°C for 5 min as previously described (23).

LOH Analysis. PCR products were electrophoresed on 6% denaturing polyacrylamide gel containing 7.7 M urea at 1600V for 2 h. Genomyx SC scanner (Beckman Coulter, Fullerton, CA) was used to image the fluorescent-labeled PCR products and densitometric analysis was performed with ClaritySC software (Media Cybernetics, Silver Spring, MD). Intensity calculations and comparisons of the specific

alleles in patients' normal control and respective BM DNA were performed to evaluate for LOH. The LOH was defined if a greater than 50% reduction of intensity was noted in one allele from tumor or BM DNA when compared with the respective allele in the matched-paired lymphocytes (23).

Clinical and histopathologic data was obtained from patient chart review and the Breast Tumor Registry at the John Wayne Cancer Institute. Chi-Square and Wilcoxon Rank Sum tests were performed for statistical evaluation of association of BM LOH status and known prognostic parameters.

RESULTS

DNA from BM aspirate plasma samples were isolated and purified. Eight microsatellite markers representing six chromosomes known to have frequent LOH in primary breast tumors were assessed. LOH was identified in 11 (23%) of 48 patients' BM aspirates. LOH was most commonly identified at microsatellite marker D14S62 occurring in 4 (12%) of 34 informative patients. Microsatellite markers demonstrating LOH at D1S228 and D14S51 occurred in 3 (8%) of 38 informative patients each, followed by LOH at D8S321 (5%), D10S197 (4%), and D17S855 (3%). No LOH was detected for microsatellite markers D16S421 and D17S849 (Table 1). Eight of the 11 patients with detectable LOH in their BM demonstrated this event at only one of the chromosome loci assessed and three patients (1 stage I, 2 stage II patients) contained LOH for two microsatellite markers. No LOH was detected for any of the microsatellite markers assessed in the patient with DCIS or the BM aspirates collected from five healthy female donors.

There was an increased association between the presence of LOH in the BM and advanced disease stage. Six (19%) of 32 AJCC stage I patients demonstrated LOH for at least one marker, in contrast to 4 (31%) of 13 AJCC stage II patients, and 1 (50%) of 2 AJCC stage III patients (Table 2). Ten clinicopathologic prognostic factors were assessed for correlation with BM LOH status: histologic tumor type, size, grade, Bloom-Richardson score, lymph node involvement, presence of lymphovascular invasion in the primary tumor, receptor status (estrogen, progesterone, HER2) and p53 status. There was an association between larger tumor size and BM LOH positivity: 2.46 cm versus 1.81 cm, mean tumor sizes, respectively. There was also a trend towards an increased incidence of BM LOH in lymph node positive patients as compared with lymph node negative patients: 3 (33%) of 9 patients versus 8 (21%) of 38 patients, respectively. No significant correlation existed between any prognostic factor and BM LOH status in this pilot study except histology. Lobular carcinomas were more likely associated with increased LOH in BM aspirates than infiltrating ductal tumors: 6 (60%) of 10 patients versus 5 (14%) of 37 patients, respectively (Chi-Square test P=0.006). Larger populations with long-term follow-up are warranted to evaluate the clinical and prognostic utility of this assay.

To determine whether a correlation existed between the LOH detected in patients BM and their primary tumor, DNA was isolated from primary tumors and evaluated with identical microsatellite markers. Ten of the eleven patients demonstrating LOH in their BM had primary tumors available for assessment. In all ten patients the LOH identified in the BM was also present respectively in the primary tumor (Fig. 1).

Conventional histologic analysis of all specimens using standard H&E staining did not demonstrate occult tumor cells in any of the BM samples.

DISCUSSION

There is mounting evidence to suggest that the presence of occult tumor cells in the BM of breast cancer patients may have prognostic significance (9, 33-38). Furthermore, some have shown these findings to be independent of pathologic lymph node status (8, 9). These studies are important because historically 20% of lymph node negative patients will subsequently develop systemic disease and therefore early detection of BM micrometastasis may identify high-risk patients for additional systemic therapy. More so, BM provides a readily accessible source to serially monitor subclinical disease progression and the potential impact of adjuvant therapies early in the disease course. Conventional histologic analysis of BM aspirates for tumor cells has proven unreliable (3, 4). More recently, immunocytochemical techniques using antibodies to epithelial antigens expressed on tumor cells have improved detection sensitivity. However, assay reliability has been shown to be highly dependent on the antibody selected as well as the variability by which the tumor cell expresses the preferred epitope (8, 11). Finally, sample processing and antibody staining require considerable attention to methodology and an experienced reviewer to interpret the results.

With the implication of an accrual of aberrant genetic events in tumor development and progression, and their potential for clonality, these genetic markers may provide unique surrogates for monitoring subclinical disease events; particularly in light of the ease and widespread use of PCR techniques. Studies have demonstrated the

presence of circulating nucleic acids in the plasma and serum of patients with various malignancies (32). In breast cancer, LOH presence in plasma/serum has been described to occur anywhere from 15% to 66% (19, 21-23, 30). These results may vary due to differences in techniques of sample collection and processing, DNA isolation, PCR methods and scoring of LOH. Furthermore we have shown that the presence of circulating tumor DNA increases with advancing stage of disease (23). More so, because blood is rapidly circulating and an inhospitable environment, and BM is a frequent site of breast cancer relapse, we sought to determine whether BM aspirates may harbor tumor-specific DNA alterations associated with early breast cancer progression.

In this study we have developed a highly sensitive method of detecting tumor-specific DNA in the BM aspirates of breast cancer patients. The increased incidence in more advanced stages correlates with tumor burden and therefore may have applicability as a surrogate marker for disease detection, prognosis and monitoring tumor progression and response to therapy. Although there was an association between known prognostic factors in breast cancer (tumor size, lymph node status, and AJCC stage) and BM LOH in this initial report, further studies are underway to address these findings in a larger cohort of patients for clinical significance. The advantage of this approach is its ease of use as an assessment tool and its broad application to a variety of malignancies. Identification of additional tumor-specific genetic markers or combinations thereof may further enhance the assay's utility. Stable tumor-specific DNA markers that accurately reflect minimal disease states, correlate with tumor progression and which can be readily measured are highly desired. The genetic-based diagnostic test we have described is highly sensitive and specific. This approach may provide a unique

alternative/supplement to optical systems for occult tumor detection which can be technically demanding and viewer dependent, or those methods (RT-PCR) that assess mRNA markers which may have limited specificity as a result of unstable gene products, variable expression levels and nonspecific transcripts (39-42). Although the source of this free DNA is unknown (i.e.: tumor cells in BM, blood or the primary tumor), detection of genomic alterations in BM can be significant and may prove more specific than immunohistochemical and/or current mRNA marker assays. Larger studies are presently underway to evaluate these findings and determine clinicopathologic correlations.

We found LOH on chromosome 14q as the most frequent event identified on circulating DNA in BM. In a previous study LOH on 14q has been shown to occur more commonly in primary tumors without lymph node metastasis suggesting a site for a possible metastasis related gene, however the metastasis itself was not assessed for LOH (43). One explanation is that metastatic clones at different sites may demonstrate different LOH profiles. Additionally, differences in these results may reflect the stability of this marker as detected from various sources (blood, BM, tumor tissues) or it may be uniquely associated with site-specific metastasis. Molecular markers that are specific for the metastatic phenotype and/or sites of metastasis may prove useful for focusing clinical assessments.

Our incidence of LOH ranged from 0-12% for any of the microsatellite markers for informative cases. Similar detection of LOH has been described from the peripheral plasma/serum of early stage breast cancer patients (19, 22, 23). For 10 of the 11 patients who's BM contained LOH, primary tumor blocks were available for assessment and in all

cases a similar corresponding LOH pattern was identified in the respective primary tumor specimens. The findings demonstrate the specificity of this marker detection system. Moreover, no patients had detectable tumors cells identified on routine histopathologic examination. This demonstrates the relative ease and sensitivity DNA detection assays pose as potential surrogates of subclinical disease. Because of earlier detection of breast cancers and the benefits of adjuvant chemotherapy in these stages the implications of these findings will require long-term follow-up for clinical correlation. However, it must be emphasized that a need exists for improved methods of occult disease surveillance to more accurately assess individual patient risk and modify treatment strategies accordingly before clinical manifestations occur.

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TABLE 1: LOH frequency in Breast Cancer Patients' Bone Marrow Aspirates

Microsatellite marker	LOH in BM aspirates/informative cases (%)
D14S62	4/34 (12%)
D14S51	3/38 (8%)
D1S228	3/38 (8%)
D8S321	2/39 (5%)
D10S197	1/26 (4%)
D17S855	1/37 (3%)
D17S849	0/31 (0%)
D16S421	0/28 (0%)

TABLE 2: Association of LOH in patients' BM aspirates with AJCC stage

AJCC Stage	Patients with LOH in BM/ total patients (%)
I	6/32 (19%)
II	4/13 (31%)
III	1/2 (50%)

Figure Legend

Fig. 1 Representative images demonstrating LOH in breast cancer patients' paired BM aspirate (BM) and primary tumors (T) at D14S62, D14S51, and D8S321, respectively. Allelic loss is represented by the arrows. First lane of each panel exhibits patients' lymphocyte DNA (L) allele pattern as a control.

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Detection of Occult Metastatic Breast Cancer Cells in Blood by a Multimolecular Marker Assay: Correlation with Clinical Stage of Disease[1]

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ABSTRACT

Currently, molecular markers offer the unique opportunity to identify occult metastasis in early stage cancer patients not otherwise detected with conventional staging techniques. To date, well-characterized molecular tumor markers to detect occult breast cancer cells in blood are limited. Because breast tumors are heterogeneous in tumor marker expression, we developed a "multimarker" reverse transcription-PCR assay combined with the highly sensitive electrochemiluminescence automated detection system. Breast cancer cell lines (n = 7), primary breast tumors (n = 25), and blood from normal donors (n = 40) and breast cancer patients [n = 65; American Joint Committee on Cancer (AJCC) stages I-IV] were assessed for four mRNA tumor markers: β-human chorionic gonadotropin (β-hCG), oncogene receptor (c-Met), β 1→4-*N*-acetylgalactosaminyl-transferase, and a tumor-associated antigen (MAGE-A3). None of the tumor markers were expressed in any normal donor bloods. Breast cancer cell lines and primary breast tumors expressed β-hCG, c-Met, β 1→4-*N*-acetylgalactosaminyl-transferase, and MAGE-A3 mRNA. Of the 65 breast cancer patient blood samples assessed, 2, 3, 15, 49, and 31% expressed 4, 3, 2, 1, and 0 of the mRNA tumor markers, respectively. At least two markers were expressed in 20% of the blood specimens. The addition of a combination of markers enhanced detection of systemic metastasis by 32%. In patient blood samples, the MAGE-A3 marker correlated significantly with tumor size (P = 0.0004) and AJCC stage (P = 0.007). The combination of β-hCG and MAGE-A3 mRNA markers correlated significantly with tumor size (P = 0.04), and the marker combination c-Met and MAGE-A3 showed a significant correlation with tumor size (P = 0.005) as well as AJCC stage (P = 0.018). A multimarker reverse transcription-PCR assay that correlates with known clinicopathological prognostic parameters may have potential clinical utility by monitoring tumor progression with a blood test.

INTRODUCTION

Recurrent disease in breast cancer patients is a consequence of undetected metastasis that has occurred before initial diagnosis. Clinical studies have demonstrated no significant increase in the incidence of distant metastasis in patients whose lymph nodes are not removed until they develop clinical evidence of disease, suggesting that the hematogenous route is a significant source for tumor dissemination (1, 2). Therefore, blood analysis offers an appealing approach for the detection of occult metastasis in early stage patients, which may be of prognostic significance, and may provide an innovative method to monitor tumor progression and assess response to treatment.

Several molecular markers for the detection of occult breast cancer tumor cells in peripheral blood have been described (3–12). Commonly assessed mRNA markers include CK18,[3] CK19, CK20, Mucin-1 (MUC-1), and carcinoembryonic antigen. However, recent studies have shown several of these markers to be expressed in normal cells of peripheral blood, lymph nodes, and/or bone marrow yielding false-positive results (13–16). More so, many of these molecular markers are also expressed frequently in normal epithelial cells. In addition, we have shown that no one tumor marker is consistently and specifically expressed by all of the primary tumors for a particular malignancy, and marker expression may vary between a primary tumor and its metastasis (16, 17). These findings may contribute to the lack of consistent correlations between any one tumor marker and well-known clinical and pathological prognostic factors. Currently there is no consensus recommendation for the routine use of molecular markers in monitoring disease detection in blood or other body fluids (18). These conclusions are based on inconsistent results from studies on breast cancer tumor marker expression and disease detection and/or progression (3, 13, 16). Because of these limitations many investigators are developing new methods for the detection of circulating occult tumor cells.

Our hypothesis is that a multimarker approach with a panel of tumor-specific mRNA markers may improve the sensitivity and specificity for the detection of circulating tumor cells over single marker assays in patients with breast cancer. Breast tumors are composed of a heterogeneous collection of cells with differing levels of individual gene expression; therefore, the predominant cell type or its metastasis may not express a particular marker assayed (19, 20). Furthermore, tumors continuously evolve genetically over time in response to host pressures and treatment interventions, which suggests that single marker testing may be an overly simplistic method to monitor cancer progression. In this study we used a combination of tumor-specific mRNA markers to avoid the inherent limitations associated with the single marker technique. In addition, ECL was used, which we have shown allows for the quantitation of PCR results to additionally enhance assay sensitivity (21).

The study panel consisted of four mRNA tumor markers: β-hCG, c-Met, GalNAc-T, and MAGE-A3, all shown previously to be significantly expressed in breast cancer. β-hCG is a subunit of human chorionic gonadotropin, and the abnormal elevation of its expression has been demonstrated in several types of carcinomas including breast cancer (5, 22, 23). The pathophysiological mechanism of abnormally elevated β-hCG expression in carcinomas is still unknown. The c-Met oncogene encodes for hepatocyte growth factor receptor, which is a tyrosine kinase transmembrane protein receptor (24). Hepatocyte growth factor binding to the receptor transduces a signal for cell dissociation, motility, and mitogenesis (25, 26). Its expression in breast cancer has been shown recently to independently predict survival (27). GalNAc-T is a key enzyme in the biosynthetic pathway of gangliosides GM2/GD2, which

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[3] The abbreviations used are: CK, cytokeratin; β-hCG, β chain of human chorionic gonadotropin; c-Met, hepatocyte growth factor receptor; ECL, electrochemiluminescence; ECL U, ECL Units; GalNAc-T, β1→4-*N*-acetylgalactosaminyl-transferase; MAGE-A3, tumor-associated antigen; AJCC, American Joint Committee on Cancer; PBL, peripheral blood lymphocyte; PBGD, porphobilinogen deaminase; RT-PCR, reverse transcription-PCR.

are oncofetal glycolipids found elevated in expression on the surface of various types of cancer cells (28–30). GalNAc-T can add specific carbohydrate molecules to other carbohydrate molecules as well as proteins; in particular, GalNAc-T adds specific carbohydrate molecules to GM3 and GD3 to produce GM2 and GD2, respectively (29). Gangliosides have been shown to play a role in tumor progression (29–31). GM2 has been found on breast cancer cells (32, 33). MAGE-A3 has been found to be expressed in several types of carcinomas including breast cancer and encodes for a highly immunogenic protein with an unknown function (34–36). The only normal tissues as assessed by molecular techniques that express this gene are testis and placenta. In this study we demonstrate the expression of these RT-PCR markers in breast cancer tumors and their potential clinicopathological utility in assessing patients blood.

PATIENTS AND METHODS

Cell Lines. The breast cell lines BT-20, MCF-7, T-47D, and MDA-MB-231m and the choriocarcinoma cell line JAR were obtained from the American Type Culture Collection (Rockville, MD) and cultured according to instructions. In addition, the 734B line is an established subclone of MCF-7 (5). The breast cancer cell lines JM992Br and PM277Br are breast cancer cell lines established and characterized at the John Wayne Cancer Institute. The cell lines were grown in RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus 10% FCS (heat-inactivated), penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY) in T75 cm^2 flasks.

Surgical Specimens and Blood Preparation. Breast tumor surgical specimens were obtained after informed consent in consultation with the surgeon and pathologist. The patients were from two hospitals: Saint John's Health Center in Santa Monica, CA, and the Martin Luther King, Jr./Drew Medical Center in South Central Los Angeles, CA. All of the tissues were collected and dissected under stringent sterile conditions to prevent RNA contamination. Institutional review board approval was obtained for the use of human subject blood specimens from breast cancer patients and normal healthy donors. Blood (10 ml) was collected in sodium citrate-containing tubes, as described previously (16), from breast cancer patients (stages I, II, and III before initial surgery, and stage IV at diagnosis) and healthy female donor volunteers. The PBLs were collected using Purescript RBC lysis buffer (Gentra, Minneapolis, MN), and centrifugation followed by physiological PBS wash.

TRI Reagent (Molecular Research Center, Cincinnati, OH) was used to isolate total RNA from the cell lines, surgical tumor specimens, and blood lymphocytes, as described previously (16). The concentration, purity, and amount of total RNA were determined by UV spectrophotometry. The integrity of all of the RNA samples was verified by performing RT-PCR for the housekeeping gene, PBGD, and mRNA expression was assessed by ECL. Tissue processing, RNA extraction, RT-PCR assay set-up, and post-PCR product analysis were carried out in separate designated rooms.

Multimarker Detection Assay

Primer and Probe Synthesis. Biotinylated oligonucleotide primers for PCR and tris(2,2′-bipyridine)ruthenium(II) (TBR)-labeled probe for hybridization were synthesized by The Midland Certified Reagent Company (Midland, TX). Primers and probe sequences were designed for optimal PCR and ECL assay system by using Oligo Primer Analysis Software, version 5.0 by National Biomedical Systems (Plymouth, MN). To avoid amplification of genomic DNA, primers were designed to target cDNA amplification by selecting gene-specific primer sequences on different exons. The tumor marker pair sequences all spanned at least one intron, as described previously. Primer sets for tumor markers were designed for optimal activity using the ECL system.

Hybridization internal probe sequences extending through at least one exon-exon junction were selected to ensure detection of only RT-PCR specific cDNA products. This prevented nonspecific detection of corresponding genomic regions under optimal ECL conditions. Internal probes were labeled with TBR and synthesized by The Midland Certified Reagent Company. The following probe sequences were synthesized: PBGD, 5′-TBR-GTA TGC GAG CAA GCT GGC TCT TGC GG-3′; β-hCG, 5′-TBR-GCA GAG TGC ACA TTG ACA GCT-3′; c-Met, 5′-TBR-ACT TCA TAT AAG GGG TCT GGG C-3′; GalNAc-T, 5′-TBR-GTT GTA CTG GGC TCC CTG GGG T-3′; and MAGE-A3, 5′-TBR-ACT CGG CAG CAG GCA CCT CCC CCA G-3′.

RT-PCR. Reverse transcription was performed using Moloney murine leukemia virus reverse-transcriptase (Promega, Madison, WI). The same amount of RNA was used in all of the reactions for all of the samples in the study, including the control samples. All of the reverse transcription reactions were carried out with oligo-dT priming, as described previously (5). The PCR conditions were set up as follows: 1 cycle of denaturing at 95°C for 5 min followed by 35 cycles of 95°C for 1 min; 55°C for c-Met and MAGE-A3; 65°C for β-hCG and GalNAc-T for 1 min; and 72°C for 1 min before a final primer sequence extension incubation at 72°C for 10 min. PCR products were evaluated by automated ECL analysis on the ORIGEN Analyzer (IGEN International Inc., Gaithersburg, MD).

ECL Analysis of cDNA Products. PCR products were detected using an Origen Analyzer (IGEN). Briefly, 5 μl of PCR product were hybridized with 10 pmol of TBR-labeled internal probe (The Midland Certified Reagent Company) in PCR buffer (10 mmol/L Tris HCl, pH 8.3; 50 mmol/L KCl) for 30 min by denaturing at 95°C for 10 min followed with 20 min of hybridization incubation at 55°C for c-Met and MAGE-A3, and 65°C for β-hCG and GalNAc-T. The cDNA-probe hybrids were then captured by M-280 streptavidin-coated Dynabeads by vortexing at room temperature for 30 min. The Origen Analyzer was used to measure the ECL activity generated by the electrochemical reaction of the TBR and ORIGEN assay buffer (IGEN International Inc.). Results were expressed as ECL U, and positive samples were determined when the sample expresses a level of ECL U greater than the cutoff point. The cutoff point for determining sample positivity was three SDs above the mean ECL U of the healthy donor PBL samples assessed in each assay. For each assay, at least two positive controls (cell lines tested positive for the respective markers), at least three healthy donor PBL samples, and reagent-negative controls (reagent alone without RNA or cDNA) for the RT-PCR/ECL assay were included. Each assay contained its own set of controls to establish background levels for the RT-PCR/ECL system. Assay results shown in figures were representative samples from individual assays. Assays were repeated at least twice to verify results. Accuracy of the RT-PCR/ECL detection assay for PCR cDNA products has been demonstrated previously by gel electrophoresis, and Southern blot with specific cDNA probes and sequencing of the final products (5, 21, 29, 37–39).

Statistical Methods. To investigate the association between single and multimarker combinations and clinicopathological parameters, *t* test was used for continuous variables, χ^2 test was used for categorical variables, and Kruskal-Wallis test was used for ordinal variables. A two-tailed $P < 0.05$ was considered statistically significant to reject the null hypothesis.

RESULTS

Breast Cancer Cell Lines and Primary Breast Tumor Analysis. The choriocarcinoma cell line, JAR, and seven breast cancer cell lines were assessed for tumor mRNA marker expression. Three mRNA tumor markers, β-hCG, c-Met, and GalNAc-T (Fig. 1, *A–C;* Table 1), were expressed in 7 of 7 (100%) breast cancer cell lines and the JAR cell line. MAGE-A3 mRNA (Fig. 1*D;* Table 1) was expressed in 3 of 7 (43%) breast cancer cell lines and also the JAR cell line. In addition, 25 primary breast tumor specimens were evaluated for mRNA marker expression (Table 1). Of these, β-hCG mRNA was expressed in 19 of 25 (76%), c-Met mRNA in 19 of 25 (76%), MAGE-A3 mRNA in 12 of 25 (48%), and GalNAc-T mRNA in 11 of 25 (44%). By examining the number of markers detected, 24 of 25 (96%) of the breast tumors expressed at least one marker, 21 of 25 (84%) expressed at least two markers, 12 of 25 (48%) expressed at least three markers, and 4 of 25 (16%) expressed all four of the markers (Table 2). This demonstrated the heterogeneity of marker expression by a primary tumor and the potential need to use a multimarker approach for detecting occult metastatic tumor cells.

RT-PCR Marker Expression in Blood. Blood was obtained from 40 healthy female donors with no history of cancer. None of the four mRNA markers, β-hCG, c-Met, GalNAc-T, or MAGE-A3, was expressed in any of these healthy donor bloods under the optimal assay

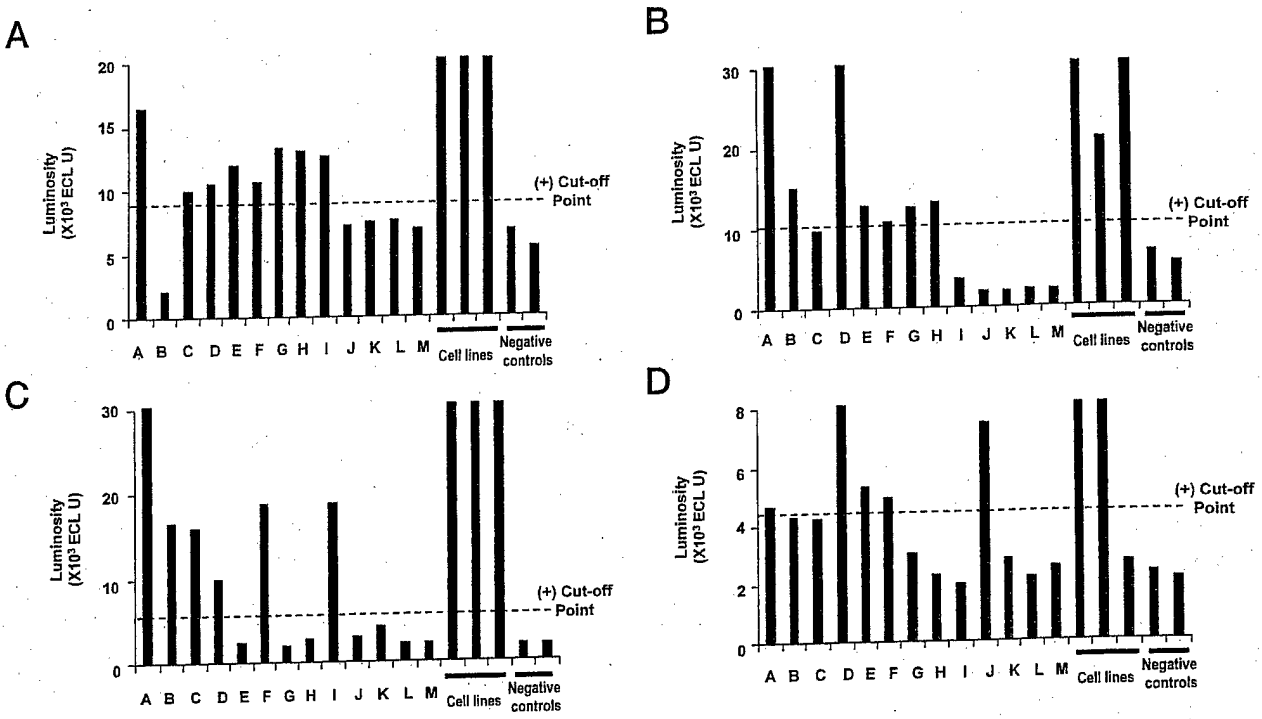

Fig. 1. Representative examples of RT-PCR/ECL analysis of individual mRNA tumor markers. The values are in 10^3 ECL U. Positive sample cutoff point is 3 SD above the mean ECL U of healthy donor PBL samples. Representative samples (same specimens for each marker) are as follows: *A–E*, breast tumor tissues; *F–J*, breast cancer patient bloods; and *K–M*, healthy donor PBL. Also shown are three breast cancer cell lines and two reagent negative controls. *A–D* are β-hCG, c-Met, GalNAc-T, and MAGE-A3 mRNA marker analysis, respectively.

conditions. All 40 of the healthy donor blood samples expressed background ECL less than the cutoff point, which was determined by adding three SDs to the mean of all 40 of the samples. Using the positive cutoff point, patient blood sample ECL U values were considered statistically significant above healthy donor samples ($P < 0.05$).

Blood was obtained from 65 breast cancer patients with an average age of 56 years (range, 29–83 years of age). Patient AJCC stage at the time of blood collection was as follows: 26 patients, stage I; 23 patients, stage II; 9 patients, stage III; and 7 patients, stage IV (Table 3). Breast cancer patient demographics are summarized in Table 4. All of the blood specimens were collected preoperatively for patients in AJCC stages I, II, and III. Stage IV patient blood specimens were collected at the time of diagnosis. Samples were coded before being assessed; individuals performing the assays were blinded from patient clinical status.

Table 1 *mRNA tumor marker expression*[a]

Tumor mRNA marker	Breast cancer cell lines ($n = 7$)	Primary breast tumors ($n = 25$)	Breast cancer patient bloods ($n = 65$)	Healthy female donor bloods ($n = 40$)
β-hCG	7 (100%)	19 (76%)	22 (34%)	0 (0%)
c-Met	7 (100%)	19 (76%)	7 (11%)	0 (0%)
GalNAc-T	7 (100%)	11 (44%)	24 (37%)	0 (0%)
MAGE-A3	3 (43%)	12 (48%)	9 (14%)	0 (0%)

[a] Marker expression refers to RT-PCR/ECL positive. All specimens assessed were PBGD RT-PCR positive by ECL. RNA (1 μg) used for each assay.

Table 2 *Distribution of mRNA tumor markers*[a]

Specimens	Number of mRNA markers positive				
	0	1	2	3	4
Breast cancer cell lines ($n = 7$)	0	0	0	4	3
Breast primary tumors ($n = 25$)	1	3	9	8	4
Breast cancer patient blood ($n = 65$)	20	32	10	2	1

[a] Specific mRNA markers assessed were: β-hCG, c-Met, GalNAc-T, and MAGE-A3 by RT-PCR/ECL.

Table 3 *Correlation of mRNA tumor markers detected in blood to AJCC stage*[a]

AJCC stage	Number of markers detected in blood					Total patients
	0	1	2	3	4	
I	11	12	3	0	0	26
II	5	12	5	1	0	23
III	2	6	0	0	1	9
IV	2	2	2	1	0	7
Total	20	32	10	2	1	65

[a] Markers detected are β-hCG, MAGE-A3, c-Met, and GalNAc-T mRNA by RT-PCR/ECL.

At least one tumor marker was detected in 69% of the blood samples (45 patients; Table 3). Two or more tumor markers were expressed in 20% (13 patients), and three or more markers were detected in 5% of patients. The most frequently expressed marker detected in the blood of patients was GalNAc-T mRNA ($n = 24$ patients; 37%; Table 1). This was followed by β-hCG mRNA ($n = 22$ patients; 34%); MAGE-A3 mRNA ($n = 9$ patients; 14%); and c-Met mRNA ($n = 7$ patients; 11%; Table 1).

Thirty-two of 65 (49%) patient blood samples demonstrated the presence of only one marker. Two markers were detected in blood samples from 10 patients (15%). Three markers were detected in blood samples from two patients (3%) and one patient blood sample expressed all four markers (2%). The most commonly coexpressed two-marker combination was GalNAc-T and β-hCG mRNA occurring in 11 patients (17%) followed by c-Met and β-hCG mRNA in four patients (6%).

Assay Detection Sensitivity. Serial RNA dilution analysis was used to assess detection sensitivity of the assay. The JAR cell line was used, because it expressed all four of the tumor markers by RT-PCR/ECL. Total RNA was isolated and purified from the cancer cell line

Table 4 *Patient clinical characteristics*

Patient characteristic	No.
Primary tumor	
T1 (≤2 cm)	35
T2 (>2 cm–5 cm)	18
T3 (>5 cm)	7
T4	2
Unknown	3
Regional lymph nodes	
Negative	32
Positive	29
Unknown	4
Distant metastasis	
M0	58
M1	7
AJCC stage	
I	26
II	23
III	9
IV	7
Histologic grade (modified Bloom-Richardson score)	
Low (3–4)	19
Intermediate (5–7)	20
High (8–9)	24
Unknown	2
Estrogen receptor	
Present	50
Absent	12
Unknown	3
Progesterone receptor	
Present	37
Absent	24
Unknown	4
Her2 receptor	
Low	41
Intermediate	3
High	6
Unknown	15
Age (yr.)	
Average	56
Range	29–83
Menopausal status	
Pre-menopausal	17
Post-menopausal	40
Unknown	8
Histologic type	
Infiltrating ductal	53
Infiltrating lobular	7
Other	4
Unknown	1
S phase	
High	13
Intermediate	6
Low	29
Unknown	17
Ploidy	
Aneuploid	20
Diploid	30
Tetraploid	2
Unknown	13

and then serially diluted in molecular biology grade water (DNase-free and RNase-free; 5 prime to 3 prime, Inc., Boulder, CO). RT-PCR/ECL was performed for each marker on serially diluted RNA. Expression of each marker could be detected at a total RNA concentration of ≥1.0 ng/ml (Fig. 2).

To additionally assess the sensitivity of the assay, an *in vitro* model was set up by serially diluting tumor cells in healthy donor PBL. Breast cancer cell lines MCF-7, T-47D, and JAR were serially diluted in healthy donor PBL to determine the cellular level of detection sensitivity. Tumor cells could be detected at concentrations of approximately 1–5 tumor cells in 10^7 PBL for individual mRNA tumor markers.

Multimarker RT-PCR Correlation with Clinicopathology. Twelve clinicopathological parameters (age, tumor grade, histological type, modified Bloom-Richardson score, DNA ploidy, S phase, primary tumor size, presence of lymph node metastasis, estrogen receptor, progesterone receptor, Her2 receptor status, and AJCC stage) were assessed to determine whether any correlation existed between mRNA tumor marker expression and well-established prognostic factors (Table 4).

Statistical analysis was performed to determine whether the frequency of RT-PCR marker expression or the combination thereof correlated with those commonly assessed clinicopathological parameters associated with breast cancer prognosis. Only tumor size and AJCC stage correlated with single- and multimarker expression. Using univariate analysis, only the MAGE-A3 marker demonstrated a significant correlation with known prognostic clinicopathological parameters. The mean tumor size in MAGE-A3 mRNA-positive patients was 4.2 ± 1.9 cm *versus* 2.2 ± 1.6 cm in MAGE-A3-negative patients ($P = 0.004$). When assessed for patient T status, MAGE-A3 marker detection significantly correlated ($P < 0.0004$) with an increasing T size as follows: 0 of 35 T_1 patients; 3 of 18 T_2 patients; 4 of 7 T_3 patients; and 1 of 2 T_4 patients were MAGE-A3 marker positive. These findings remained statistically significant in multivariate analysis ($P = 0.02$). Patients with axillary lymph node metastasis were more likely to have circulating tumor cells as demonstrated by an increased frequency of MAGE-A3 positivity (5 of 29 patients; 17%) when compared with those without lymph node involvement (3 of 32 patients; 9%). Because of the limited number of patients with lymph node metastasis, this difference did not reach statistical significance. MAGE-A3 expression was significantly ($P = 0.007$) associated with advanced AJCC stage. None of 26 patients in stage I and three of 23 patients (13%) in stage II expressed MAGE-A3, whereas 6 of 16 (38%) patients in stage III and IV were MAGE-A3 marker positive.

Combinations of markers were assessed to determine whether the frequency of any specific combinations correlated with any of the clinical or pathological parameters. The combination of c-Met and MAGE-A3 markers correlated with an increasing T classification ($P = 0.005$), as well as advanced AJCC stage ($P = 0.018$). The two marker combination of β-hCG and MAGE-A3 showed significant correlation between expression and increasing tumor size ($P = 0.04$). Two combinations of three markers c-Met, GalNAc-T, MAGE-A3, and c-Met, β-hCG, MAGE-A3 showed significant correlation with AJCC stage ($P = 0.02$) and tumor size ($P = 0.02$), respectively.

DISCUSSION

Increased accuracy in staging breast cancer patient disease and initiation of earlier therapeutic interventions are beneficial consequences of technological advancements that identify high-risk patients early in their disease course. Blood testing provides a minimally invasive method to evaluate the presence of circulating tumor cells that may serve as indicators for assessing risk of recurrence. Current imaging techniques used to identify breast cancer metastasis often require the presence of significant tumor burden for detection. Furthermore, the procurement of sufficient tissue to confirm the diagnosis can be associated with significant morbidity and cost depending on the size and location of the lesion. Therefore, the utility of detecting tumor cells in the blood potentially offers a practical, safe, and cost-effective alternative to traditional methods of diagnosing disease recurrence and/or systemic spreading.

RT-PCR analysis is a particularly sensitive technique for the purpose of detecting occult breast cancer cells in the blood, bone marrow, and lymph nodes of breast cancer patients (6–9, 11, 12). However, the detection method and analysis of cDNA products are also very critical in interpreting results. The multimarker RT-PCR/ECL assay provides a highly sensitive automated detection assay. The unique features of this assay are that it allows capture and isolation of the specific target PCR cDNA products in the solution phase, plus the specificity is

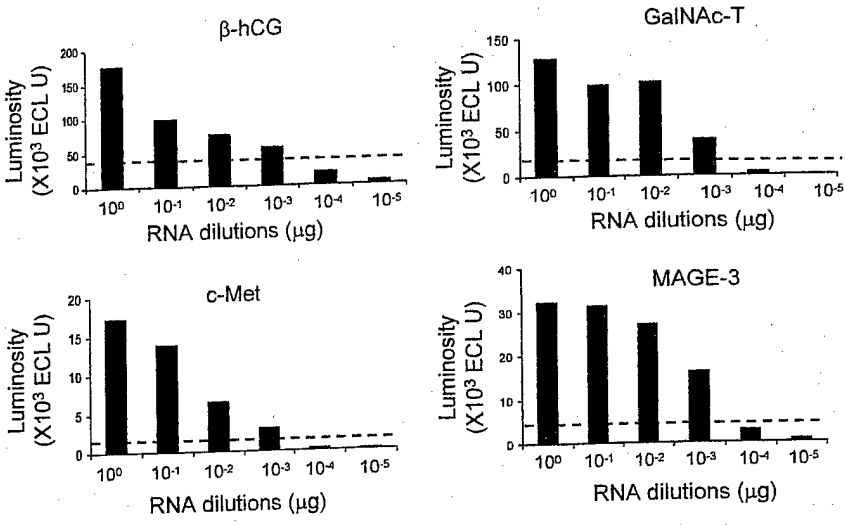

Fig. 2. Tumor cell line (JAR) serial RNA dilution. RT-PCR/ECL analysis of the four mRNA tumor markers. (---), positive detection cut-off.

enhanced by hybridization with a labeled internal probe to the specific cDNA product (21). The assay involves ECL detection using Origen instrument technology allowing comparative "semiquantitative" analysis of multiple samples. This assay may additionally enhance detection over single-marker methods by incorporating a panel of mRNA markers to account for tumor cell heterogeneity that may exist between patients, as well as between the primary tumor and its metastasis.

Using the four mRNA tumor markers β-hCG, c-Met, GalNAc-T, and MAGE-A3, we found frequent expression in breast cancer cell lines and tumor specimens, and no expression in blood from normal donors. Not all of the tumors expressed each marker, with individual marker expression in tumor specimens ranging from 44 to 76%. The higher expression in cell lines and the variable expression in tumor specimens were expected because of heterogeneity of cells within tumors. Assessing for the combination of markers improved assay sensitivity to 96%, which was 20% above the highest single-marker detection frequency. These findings provide evidence that a panel of tumor markers may increase detection over single tumor-marker techniques. Additionally, the multimarker assay can provide valuable information on the characteristic properties of tumor cells, which may more accurately gauge tumor aggressiveness as well as identify potential targets for therapy. The mRNA markers used in the assay, such as c-Met, GalNAc-T, and MAGE-A3, individually, have been demonstrated previously to be associated with tumor progression (27, 29, 31).

In this study, 45 of 65 (69%) patients had positive blood samples by RT-PCR for at least one of the four mRNA markers tested. The blood level of expression for each marker ranged from 11 to 37%, demonstrating that no one marker alone was ideal for detecting occult circulating tumor cells. By using multiple markers, the assay detected an increase (32%) in the number of patients with positive blood samples, demonstrating a significant improvement in the sensitivity over a single-marker assay.

Tumor progression can involve a latent period in which metastatic cells with different gene expression profiles exist. Detection of those prevailing cells may require the use of more than one marker over time. Nowhere is this more evident than in breast cancer where many patients treated for early stage disease may a have prolonged disease-free interval only to relapse years later. During this follow-up period, metastatic cells may undergo significant changes in gene expression. No one breast cancer marker has demonstrated consistent reliability in predicting relapse to date (40). Assessing more than one marker may enhance the upstaging of patients at the time of their initial diagnosis and allow for stratification into adjuvant therapy protocols according to the presence of a particular marker or the expression of a unique combination of markers. Furthermore, these detection methods may provide a more cost-effective approach by selectively instituting adjuvant therapy in patient subsets at high risk for metastatic disease based on a predefined molecular marker profile.

Because clinical outcomes in breast cancer often require a prolonged follow-up, which is still in progress for the current study, mRNA tumor marker expression was evaluated against known prognostic clinicopathological factors for correlation. Only MAGE-A3 mRNA expression showed a significant correlation with two prognostic parameters: pathological tumor size and advanced AJCC stage. As additional markers improved assay detection sensitivity, statistical analysis of multimarker combinations with clinical and histopathological factors were performed to determine whether significant correlations existed. The combination of c-Met and MAGE-A3 mRNA expression correlated with increasing tumor size and AJCC stage. The combination of β-hCG and MAGE-A3 mRNA correlated with tumor size and showed a trend with advanced AJCC stage. The three tumor marker combinations of c-Met, GalNAc-T, MAGE-A3, and c-Met, β-hCG, and MAGE-A3 correlated with advancing stage and tumor size, respectively. It must be noted that most AJCC stage IV patients receive chemotherapy, and its impact on tumor cell presence in blood needs to be additionally examined in defined treatment protocols. However, this subgroup comprised a relatively small proportion of patients ($n = 7$) in this study. The majority of patients enrolled in this study were diagnosed with early invasive breast cancer, AJCC stage I and II. The implications of detecting occult circulating tumor cells using a combination of molecular markers, which demonstrate clinical correlation, may not only enhance insight into the biological behavior of the tumor of an individual but may provide valuable prognostic information, which can be readily monitored throughout the disease course. Additionally, assessment of the functional role for these tumor markers in cancer progression may identify patients at high risk for relapse when compared with the same clinical stage patients whose tumor cells do not express these markers.

Approximately 30% of breast cancer patients who have tumor-negative lymph nodes develop recurrent disease (41). We have detected micrometastatic disease previously using RT-PCR in patients whose lymph nodes were negative by H&E and immunohistochemistry (37). The clinical implications of these findings await the results of longer-term follow-up. Blood testing for the presence of occult tumor cells may provide a complementary technique to enhance current methods for staging disease. The potential advantage of a blood RT-PCR assay is that it provides the

opportunity to serially assess tumor progression and/or response to therapy through an easily accessible route that is associated with minimal inconvenience to the patient.

Identification of those patients with early stage breast cancer who are at high risk of recurrence based on the detection of occult circulating tumor cells has significant prognostic implications. We have identified a panel of four tumor markers useful for the detection of circulating tumor cells in the blood of breast cancer patients. In this study, those patients whose tumors expressed certain marker combinations showed a significant correlation with tumor size, a well-established poor prognostic factor (42). Recent evidence suggests that the detection of micrometastasis in the bone marrow and/or lymph nodes of cancer patients has prognostic value and can identify those at increased risk for recurrence (43–45). Long-term clinical follow-up in those patients with occult tumor cells in the blood detected by multimarker RT-PCR will determine the clinical utility of this technique.

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