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

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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 enhance assay sensitivity (21).

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, 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 assessed (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, GaINAc-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). GaINAc-T is a key enzyme in the biosynthetic pathway of gangliosides GM2/GD2, which

Received 10/9/00; accepted 10/19/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. Supported by the Ben B. and Joyce E. Eisenberg Foundation (Los Angeles), the Associates for Breast Cancer Research (Los Angeles), the Fashion Footwear Association of New York, and Grant DAMD17-96-1-6193 and DAMD17-01-1-0279 United States Army Medical Research and Material Command. Presented in part at the 35th annual meeting of the American Society for Clinical Oncology, Atlanta, GA, May 1999.

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1 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; GaINAc-T, β1–α4-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 chorocarcinoma 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 cm² 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 (Genta, Minneapolis, MN), and centrifugation followed by physiological PBS wash.

Tissue processing, RNA extraction, RT-PCR assay set-up, and post-PCR product analysis were carried out in 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 CTA AAG GGG TCT CAA TTG ACA GCT-3’; c-Met, 5’TBR-ACT TCA TAT AAG GGG TCT CAA TTG ACA GCT-3’; GalNAc-T, 5’TBR-GTT GTA CGC TGC TCT GGG TCT CAA TTG ACA GCT-3’; c-Met, 5’TBR-CTG CGC TCT GGG TCT CAA TTG ACA GCT-3’, and MAGE-A3, 5’TBR-ACT CGG CAG GAC CCT CCC CCA G3’.-hCG, c-Met, and GalNAc-T (Fig. 1, A–C, Table 1), were expressed in 3 of 7 (43%) breast cancer cell lines and the JAR cell line. MAGE-A3 mRNA (Fig. 1D; 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. 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. 1D; 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 conditions.
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.

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 $\beta$-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 $\beta$-hCG mRNA occurring in 11 patients (17%) followed by c-Met and $\beta$-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...
and then serially diluted in molecular biology grade water (DNase-free and RNase-free; S 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 T1 patients; 3 of 18 T2 patients; 4 of 7 T3 patients; and 1 of 2 T4 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
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 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 multi-marker RT-PCR will determine the clinical utility of this technique.

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Cancer Res 2001;61:8845-8850.

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