Detection of Circulating Tumor Cells in Early-Stage Breast Cancer Metastasis to Axillary Lymph Nodes

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Abstract

Purpose: Clinical and pathologic prognostic factors do not always accurately predict disease outcome. Patients with early-stage breast cancer may harbor clinically significant but undetected systemic disease. We hypothesized that a multimarker quantitative real-time reverse transcription-PCR (qRT) assay could detect circulating tumor cells (CTC) in patients with early-stage breast cancer and correlate with sentinel lymph node (SLN) and non-SLN metastasis status.

Experimental Design: Blood samples from 90 women with the American Joint Committee on Cancer stages I to III breast cancer and 39 age-matched normal healthy volunteers were assessed by qRT for mRNA expression of three markers: stanniocalcin-1 (STC-1), N-acetylgalactosaminyltransferase (GalNacT), and melanoma antigen gene family-A3 (MAGE-A3). CTC biomarker detection was correlated with overall axillary LN (ALN), SLN, and non-SLN histopathology status.

Results: CTCs were detected in 39 of 90 (43%) patients, but not in normal volunteers. At least one CTC biomarker was detected in 10 of 39 (29%) stage I patients, 19 of 42 (45%) stage II patients, and 10 of 13 (77%) stage III patients. In multivariate analysis, only lymphovascular invasion and ≥2 CTC biomarkers detected significantly correlated with ALN metastasis [odds ratio (OR), 12.42; 95% confidence interval (95% CI), 3.52-43.77, P < 0.0001; and OR, 3.88; 95% CI, 1.69-8.89, P = 0.001, respectively]. The number of CTC biomarkers detected similarly correlated with SLN and non-SLN metastasis status (P = 0.0004). At least one CTC biomarker was detected in 10 of 11 (91%) patients with non-SLN metastases.

Conclusion: The detection of CTCs offers a novel means to assess the presence of systemic disease spreading relative to SLN and ALN histopathology status.

Although several clinical and pathologic factors have been identified as having prognostic utility, the presence of axillary lymph node (ALN) metastasis remains the most important predictor of disease-free and overall survival for breast cancer (1–3). Breast cancer survival is multifactorial, however, and even patients with small tumors and no evidence of ALN metastasis may ultimately succumb to distant metastasis within 5 years (2). Isolated tumor cells have been identified in the bone marrow (BM) of up to 16% of the American Joint Committee on Cancer (AJCC) stages I and II breast cancer patients and have been shown to decrease disease-free and overall survival (4, 5). The demonstration of tumor cells in BM of AJCC stage I and II breast cancer suggests that even early stages may result in circulating tumor cells (CTC), some of which could produce clinical metastasis.

The molecular detection of circulating breast cancer cells in blood has previously been studied (4, 6–20) and can predict progression-free and overall survival (7, 8). There are multiple approaches to detecting CTCs (7, 14, 19). Of these, multimarker quantitative real-time reverse transcription-PCR (qRT) is one of the most sensitive and specific assays (14). Although the ability to detect CTCs is not new, few studies have associated the presence of CTCs with early disease progression or with known prognostic factors, such as ALN status. Most descriptions of CTCs in breast cancer have excluded patients with localized, early-stage, and nonmetastatic disease, focusing on those with clinically palpable ALN or distant metastases.

We have developed a multimarker qRT assay for the detection of CTCs in the blood of breast cancer patients with early clinically localized and early regional metastasis [sentinel lymph node (SLN) positive]. The assay uses three mRNA markers established in primary and metastatic breast cancers: stanniocalcin-1 (STC-1), N-acetylgalactosaminyltransferase (GalNacT), and melanoma antigen gene family-A3 (MAGE-A3; refs. 12, 21, 22). The use of multiple markers compensates for tumor cell heterogeneity in marker expression, low mRNA levels, and the infrequency of CTCs in blood. STC-1 is located on chromosome 8p, a region associated with the amplification in breast cancer, and functions in cellular calcium homeostasis and resistance to hypoxia (21). GalNacT is one of the key
enzymes in the biosynthetic pathway of gangliosides GM2/GD2, oncofetal glycolipids overexpressed on the surface of malignant cells, including breast cancer (22–24). MAGE-A3 is tumor-specific and commonly produced by several tumor types, including breast cancer (12, 25, 26).

We hypothesized that a blood-based multimarker qRT assay could detect CTCs in patients with early-stage breast cancer. Furthermore, we hypothesized that CTCs would be detected with greater frequency in patients with SLN and/or non-SLN metastasis and, therefore, correlate with disease progression.

Materials and Methods

Breast cancer cell lines. Breast cancer cell lines (Au565, ZR-75-1, 734/B24, MDA-MB435, HBL100, T47D, MCF7, OR-090-1, 231/45, BT-20, and ZR-75-30) and one choriocarcinoma cell line (JAR) were maintained in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. All lines were grown at 37°C in a 5% CO2 atmosphere incubator.

Patient selection. Female breast cancer patients treated at the Joyce Eisenberg-Keefer Breast Center at St. John's Health Center (SJHC) between January 2002 and May 2005 were enrolled. The study protocol was approved by the SJHC/John Wayne Cancer Institute Human Subjects Institutional Review Board. All patients participating in the study provided informed consent.

Ninety patients with invasive breast cancer undergoing resection and SLN biopsy and/or ALN dissection were enrolled. Seven of the patients did not undergo SLN biopsy. Peripheral blood was drawn immediately before surgery. Of these, 24 had paraffin-embedded (PE) primary tumors available for analysis. Patients with carcinoma in situ were excluded from the study. Patients were staged according to the AJCC manual (27). As a control group, 39 normal healthy female volunteers were enrolled. All assay investigators were blinded as to the disease status of the participants.

Blood processing. Patients had 10 mL of blood drawn into sodium citrate–containing tubes as previously described (28, 29). Blood was processed in a designated blood-processing room. Peripheral blood cells were collected with Purescript RBC Lysis Solution (Gentra; ref. 29). As a control housekeeping gene.

RNA extraction. Tri-Reagent (Molecular Research Center) was used to isolate total cellular RNA from blood samples and cell lines as previously described (12, 29). All RNA extraction was done in a designated sterile laminar flow hood with RNase-free plasticware. RNA quantity and quality were assessed by UV spectrophotometry. RNA was quantified and assessed for purity by UV spectrophotometry. RNA was maintained in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. All lines were grown at 37°C in a 5% CO2 atmosphere incubator.

Primers and probes. Primer and probe sequences were designed for the qRT as previously described (24). The forward primers, fluorescence resonance energy transfer probe sequences, and reverse primer sequences were designed as follows: STC-1 (forward), 5'-CATCTTCACACAGATACT-3', (probe) 5'-FAM-CTGCTGGAAATGATGAAGAC-AMC-BHQ-1-3', (reverse) 5'-CATGTTAGCCCACATTTC-3'; GalNaCT, (forward) 5'-CCAAACAGACACACACGT-3', (probe) 5'-FAM-ATGAGGTGTGGT-TTCAATCCGCA-BHQ-1-3', (reverse) 5'-GATTGTTACTGGCGCTCCCT-3'; MAGE-A3, (forward) 5'-AGGAGAAGACATGCGAGTG-3', (probe) 5'-FAM-AGTTTGTCAGCTTCACTATCCGCA-BHQ-1-3', (reverse) 5'-GATTGTTACTGGCGCTCCCT-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), (forward) 5'-GGTGTGAACCATGAGAAGT-3', (reverse) 5'-GATGTTACTGGCGCTCCCT-3'.

Table 1. Individual CTC biomarkers positive by qRT

<table>
<thead>
<tr>
<th>Specimen</th>
<th>STC-1</th>
<th>GalNaCT</th>
<th>MAGE-A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast cancer cell lines*</td>
<td>92% (11/12)</td>
<td>100% (12/12)</td>
<td>83% (10/12)</td>
</tr>
<tr>
<td>Primary breast tumors</td>
<td>96% (23/24)</td>
<td>75% (18/24)</td>
<td>67% (16/24)</td>
</tr>
<tr>
<td>Normal donor blood</td>
<td>0% (0/39)</td>
<td>0% (0/39)</td>
<td>0% (0/39)</td>
</tr>
</tbody>
</table>

*One cell line (JAR) is a choriocarcinoma.

Carrier for precipitation. RNA quantity and quality were assessed by UV spectrophotometry and RiboGreen (Molecular Probes).

Multimarker qRT assay. Reverse transcription reactions were done with Moloney murine leukemia virus reverse transcriptase (Promega)
and oligo(dT) primers (29). The qRT assay was done using an iCycler iQ Real-Time Thermocycler (Bio-Rad Laboratories). A total of 250 ng of RNA was used for each reaction in 96-well plates (Fisher Scientific) along with each primer, probe, and iTaq custom Supermix (Bio-Rad Laboratories). Samples were amplified with a precycling hold at 95°C for 10 min, followed by specific cycles of denaturation for each CTC marker at 95°C for 1 min, annealing for 1 min (55°C for GAPDH, 60°C for STC-1, 63°C for GalNacT, and 63°C for MAGE-A3) and extension at 72°C for 1 min. A standard curve was generated using the threshold cycle (Ct) of nine serial dilutions of plasmid templates (108-100 copies). The Ct of each sample was interpolated from the CTC marker standard curve, and the number of mRNA copies was calculated by the iCycler iQ Real-Time Detection System Software. The Ct cutoff for a negative specimen was determined by assessing 39 normal donor blood specimen and diluted tumor cells expressing the specific marker as previously described (28, 29). The highest Ct for which mRNA could not be detected in normal donor blood for an individual CTC marker was used as the cutoff value for a positive result. The cutoff threshold cycles for the CTC markers were as follows: STC-1, 37; GalNacT, 38; MAGE-A3, 40. Each assay was done in duplicate and included positive, negative (peripheral blood lymphocytes (PBL) of healthy donors), and reagent controls (reagents alone without cDNA). The mean number of mRNA copies for each gene was used for analysis.

**Statistical analysis.** To assess the ability of each individual marker to predict metastasis, we assayed blood from patients with (n = 52) and without ALN metastasis (n = 38). Univariate logistic regression was used to compare each marker or number of markers between the two ALN groups to determine its ability to predict ALN status. A P value ≤ 0.05 was considered significant. We also constructed receiver-operating characteristic (ROC) curves to evaluate the predictive power of each biomarker or number of markers. The area under the curve (AUC) was computed via numerical integration. Fisher's exact tests were used to compare categorical clinical variables between ALN metastasis-negative and positive groups. Multivariate logistic regression was used to construct a classification model to discriminate between ALN metastasis-negative and metastasis-positive groups. Forward stepwise model selection was used to build the logistic regression model, which included the number of CTC markers (≥2, 1, 0) and the known clinicopathologic prognostic factors.

**Table 3. Number of CTC biomarkers detected: correlation to metastatic ALN**

<table>
<thead>
<tr>
<th>(A) AJCC stage, n (%) *</th>
<th>Number of CTC biomarkers</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>25 (71)</td>
<td>23 (55)</td>
<td>3 (23)</td>
<td>51 (57)</td>
<td>0.0007</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>9 (26)</td>
<td>10 (24)</td>
<td>4 (31)</td>
<td>23 (26)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td></td>
<td>1 (3)</td>
<td>9 (21)</td>
<td>6 (46)</td>
<td>16 (18)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(B) Number of metastatic ALN, n (%) *</th>
<th>Number of CTC biomarkers</th>
<th>0</th>
<th>1</th>
<th>≥2</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>35 (67)</td>
<td>12 (50)</td>
<td>4 (27)</td>
<td>51 (57)</td>
<td>0.0007</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>15 (29)</td>
<td>3 (13)</td>
<td>5 (33)</td>
<td>23 (36)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td></td>
<td>2 (4)</td>
<td>8 (35)</td>
<td>6 (40)</td>
<td>16 (18)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(C) Number of metastatic ALN, n (%) *</th>
<th>Number of CTC biomarkers</th>
<th>0</th>
<th>1-3</th>
<th>≥4</th>
<th>Total</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>35 (67)</td>
<td>13 (50)</td>
<td>3 (25)</td>
<td>51 (57)</td>
<td>0.0007</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>15 (29)</td>
<td>4 (15)</td>
<td>4 (33)</td>
<td>23 (36)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td></td>
<td>2 (4)</td>
<td>9 (35)</td>
<td>5 (42)</td>
<td>16 (18)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(D) SLN status, n (%) †</th>
<th>Number of CTC biomarkers</th>
<th>SLN(-)</th>
<th>SLN(+)</th>
<th>Non-SLN(+)</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>33 (67)</td>
<td>13 (57)</td>
<td>1 (9)</td>
<td>47 (57)</td>
<td>0.0004</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>14 (29)</td>
<td>3 (13)</td>
<td>5 (45)</td>
<td>22 (27)</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td></td>
<td>2 (4)</td>
<td>7 (30)</td>
<td>5 (45)</td>
<td>14 (17)</td>
<td></td>
</tr>
</tbody>
</table>

*83 patients underwent SLN biopsy.
† All non-SLN(+) histopathology patients were SLN histopathology (+).

**Table 4. Detection of CTC biomarker and ALN status: univariate analysis**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>N</th>
<th>P</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>STC-1</td>
<td>5.83 (1.48, 22.98)</td>
<td>90</td>
<td>0.012</td>
<td>0.60</td>
</tr>
<tr>
<td>GalNacT</td>
<td>2.44 (1.01, 5.91)</td>
<td>90</td>
<td>0.048</td>
<td>0.60</td>
</tr>
<tr>
<td>MAGE-A3</td>
<td>11.53 (2.40, 55.44)</td>
<td>90</td>
<td>0.0023</td>
<td>0.64</td>
</tr>
<tr>
<td>Number of CTC biomarkers (0, 1, 2, 3)</td>
<td>2.74 (1.53, 4.89)</td>
<td>90</td>
<td>0.0007</td>
<td>0.68</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥2, 1, 0)</td>
<td>2.83 (1.54, 5.19)</td>
<td>90</td>
<td>0.0008</td>
<td>0.68</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥2, 1, 0)</td>
<td>2.83 (1.19, 6.73)</td>
<td>90</td>
<td>0.019</td>
<td>0.63</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥2, &lt;2)</td>
<td>14.58 (3.07, 69.36)</td>
<td>90</td>
<td>0.0008</td>
<td>0.67</td>
</tr>
</tbody>
</table>
Univariate logistic regression on the number of markers was also done to distinguish between SLN-only positive and SLN/non-SLN positive.

Leave-one-out cross-validation was used to validate the logistic regression model. The cross-validation strategy first removes one observation and then fits a logistic regression model from the remaining cases with all of the predictors. Stepwise model selection is used for each of these models to remove variables that do not improve the model. Subsequently, we used the predictor values based on a cutoff probability of 50% for the case that was left out to compute a predicted class for that observation. The cross-validation error rate is then the number of samples predicted incorrectly divided by the number of samples.

The Jonckheere Terpstra test was done to assess the association of ordered categorical variables, specifically the number of CTC markers with stage, the number of ALN metastasis, and overall nodal status (ALN negative, SLN positive plus non-SLN positive; ref. 30).

Results

Cell lines and tumor tissue analysis. At least two CTC biomarkers were positive in 12 of 12 (100%) breast cancer cell lines and the JAR line. With regard to individual markers, 11 of 12 (92%), 12 of 12 (100%), and 10 of 12 (83%) cell lines showed expression of STC-1, GalNacT, and MAGE-A3, respectively (Table 1). Primary breast tumors showed a high expression of all three CTC biomarkers. Specifically, 23 of 24 (96%), 18 of 24 (75%), and 16 of 24 (67%) primary breast tumors showed expression of STC-1, GalNacT, and MAGE-A3, respectively (Table 1). These studies showed the significant frequency of the mRNA markers in in vitro and in vivo breast cancer cells.

Detection of CTC correlates with tumor progression. The clinicopathologic characteristics of the patients are listed in Table 2. Of the 90 patients enrolled, 35 were AJCC stage I, 42 were stage II, and 13 were stage III. Overall, ALN metastases were present in 38 patients (42%), whereas 52 patients (58%) were node negative (all but 7 patients underwent SLN biopsy).

To determine if the CTC biomarkers could be detected in early-stage breast cancer and if detection rates paralleled disease progression, we assessed CTCs according to AJCC stage. CTC detection was significantly associated with AJCC stage \( (P = 0.0007) \). Overall, at least one CTC biomarker was detected in 39 of 90 (43%) patients, regardless of stage (Table 3). At least one CTC biomarker was detected in 10 of 35 (29%) stage I, 19 of 42 (45%) stage II, and 10 of 13 (77%) stage III patients, indicating that our panel can detect CTCs in early-stage disease and correlates well with stage. No CTC biomarker was detected in the blood of any healthy normal female volunteers under the optimal assay conditions (Table 1).

Detection of CTC predicts ALN status. There was no relation between the ALN status and age, histologic grade (well/moderate/poor), histologic type, ER status, PR status, HER2/neu expression, tumor size (≤2 cm versus >2 cm), DNA ploidy, S phase, or p53 values (Table 2). Lymphovascular invasion (LVI) and Ki-67 values were the only clinicopathologic factors to correlate with ALN metastasis \( (P < 0.001 \) and \( P = 0.047 \), respectively).

In a univariate analysis, the detection of each CTC biomarker was significantly associated with ALN metastasis [STC-1: odds ratio \( \text{OR} \) 5.83; 95% confidence interval \( \text{CI} \), 1.48-22.98, \( P = 0.012 \); GalNacT: \( \text{OR}, 2.44; 95\% \text{CI}, 1.01-5.91, P = 0.048 \); MAGE-A3: \( \text{OR}, 11.53; 95\% \text{CI}, 2.40-55.44, P = 0.002 \)]. The area under the ROC curve for these individual biomarkers was 0.60, 0.60, and 0.64, respectively (Table 4). The number of CTC biomarkers detected (≥1 or ≥2) was also significantly correlated with ALN metastasis (Table 4). Of the clinicopathologic factors, only LVI and Ki-67 achieved statistical significance \( (P = 0.0001 \) and \( P = 0.039 \), respectively).

The biomarkers that were significant in the univariate analysis and known clinical variables were included in the multivariate logistic regression model. A stepwise procedure was used for covariate selection to determine which factors were associated with ALN metastasis in breast cancer patients. Histologic type was excluded from the model because the pathologic diagnosis was infiltrating ductal carcinoma in 84 patients (93%). In 36 of 90 patients (40%), the S phase was indeterminate due to overlapping diploid and aneuploid cell populations. S phase was therefore excluded from the multivariate model. Accordingly, LVI \( (\text{OR}, 12.42; 95\% \text{CI}, 3.52-43.77, P < 0.0001) \) and ≥2
Circulating Breast Cancer Cells

Table 6. Detection of CTC biomarker and SLN status: univariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds ratio (95% CI)</th>
<th>N</th>
<th>P</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of CTC biomarkers (0, 1, 2, 3)</td>
<td>1.86 (0.88, 3.92)</td>
<td>34</td>
<td>0.1</td>
<td>0.692</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥2, 1, 0)</td>
<td>2.38 (0.97, 5.89)</td>
<td>34</td>
<td>0.06</td>
<td>0.698</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥1, 0)</td>
<td>13.00 (1.42, 119.06)</td>
<td>34</td>
<td>0.023</td>
<td>0.737</td>
</tr>
<tr>
<td>Number of CTC biomarkers (≥2, &lt;2)</td>
<td>1.90 (0.43, 8.39)</td>
<td>34</td>
<td>0.39</td>
<td>0.575</td>
</tr>
</tbody>
</table>

CTC biomarkers detected (OR, 3.88; 95% CI, 1.69-8.89, P = 0.001) were significantly correlated with ALN metastasis (Table 5).

We used a leave-one-out cross-validation to verify the logistic regression model. The leave-one-out cross-validation error rate based on logistic regression models was 18.4% (14 of 76). All (of the 76) of the models generated in the leave-one-out analysis used the same set of two predictors (lymphovascular invasion and number of CTC biomarkers). The ROC curve was computed for the logistic regression model (Fig. 1). Using a cutoff probability of 50%, we obtained a sensitivity of 81.3% and a specificity of 81.8%. The calculated area under the ROC curve was 0.84 for the logistic regression model.

In a univariate analysis for the node-positive patients, the number of markers (≥1, 0) was significantly associated with SLN status (OR, 13.00; 95% CI, 1.42-119.06, P = 0.023) and the number of markers (≥2, 1, 0) was marginally significant (OR, 2.38; 95% CI, 0.97-5.89, P = 0.060). The area under the ROC curve for the two models was 0.737 and 0.698, respectively (Table 6).

Jonckheere Terpstra test was used to assess the association of identified CTC biomarkers with the number of ALN metastasis (0 versus 1 metastatic node versus ≥2 metastatic nodes; Table 3). The study involved 90 patients that received ALN dissections (7 did not receive SLN biopsy). CTCs were detected with increasing frequency as the number of metastatic ALN increased (P = 0.0007). At least one CTC biomarker was detected in 33% of patients who had no histologic evidence of metastasis. Those patients with ≥2 ALN metastasis had at least one detected CTC biomarker in 73% of patients.

We further classified the number of positive nodes according to AJCC staging criteria: N0, metastasis negative; N1, 1-3 nodes(+); and N2, ≥4 nodes(+). CTCs were detected with increasing frequency as the number of metastatic ALN increased (P = 0.0007). At least one CTC biomarker was detected in 33% of patients who had no histologic evidence of metastasis in their ALN. Those patients with ≥4 ALN metastasis had at least one detected CTC biomarker in 75% of patients (Table 3).

Similarly, a Jonckheere Terpstra test was done to assess the association of identified CTC biomarkers with the SLN status [node(-) versus SLN(+) versus non-SLN(+)]. The number of detected CTC biomarkers was significantly associated with SLN and non-SLN metastases (P = 0.0004). At least one CTC biomarker was identified in 10 of 11 (91%) SLN patients with non-SLN metastasis (Table 3).

Discussion

If breast cancer is diagnosed when tumors are small (<1 cm), and with no metastasis to regional lymph nodes, 5-year survival exceeds 90% (31). Conversely, for large (>5-cm) breast cancers with regional nodal metastasis, the 5-year survival can be as low as 40% (31). Several clinicopathologic factors such as tumor size, histologic type, tumor grade, LVI, HER-2/neu overexpression, and hormone receptor status have been recognized as having prognostic utility (32–34).

To date, the best indicator of ALN metastasis is metastasis status in the SLN. Although the SLN biopsy is associated with fewer complications than level I and II ALN dissection, it is not without morbidity (35, 36). SLN biopsy has a low but measurable false-negative rate and provides no information regarding the presence of additional non-SLN metastasis, which may occur in 40% to 70% of cases (37–39). Therefore, it is recommended that patients who have metastatic cancer in their SLN undergo a completion level I and II ALN dissection, which may be associated with upper extremity lymphedema, wound complications, or nerve injury in a significant proportion of patients. Newer, more accurate, and less invasive means of predicting ALN metastasis would greatly improve patient management. In the setting of confirmed SLN metastasis, the same blood-based assay may help clinicians assess the likelihood of additional metastatic disease in the remaining ALN, and the potential of systemic disease spreading. Our current study supports the detection of CTCs in breast cancer patients to predict the potential risk of ALN metastasis.

In a univariate analysis, each of the three biomarkers significantly predicted ALN status, as did LVI and Ki-67. In multivariate analysis, no single CTC biomarker significantly predicted ALN status, but the detection of ≥2 CTC biomarkers was the only factor other than LVI that significantly predicted ALN metastasis in a multivariate model.

Because we do not have prolonged clinical follow-up regarding the disease-free and overall survival of these patients, it is not possible at this time to determine if these represent false-positive results or true systemic metastases that are, as of yet, clinically occult. The assay could distinguish AJCC N0 from AJCC N2 patients with ≥4 metastatic nodes. N2 patients have significantly worse outcomes than node-negative patients or N1 patients and may benefit from adjuvant axillary radiation in addition to a complete ALN dissection (1, 40).

A limitation of the SLN biopsy is its inability to provide information regarding the status of non-SLN, which may be harboring metastatic disease (41). Currently, SLN(-) patients may be observed, whereas those who are SLN(+) are treated with a completion level I and II ALN dissection. An assay that can predict the risk of metastasis spread beyond the SLN would be of important utility in disease treatment. Our assay did well in this regard, accurately predicting the status of non-SLN in 91% of cases.

The multimarker CTC assay correlated well with disease progression, paralleling AJCC stage. These findings suggest that the assay may have clinical utility as a detection marker for
References
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