Cytokeratin-19 and Mammaglobin Gene Expression in Circulating Tumor Cells from Metastatic Breast Cancer Patients Enrolled in North Central Cancer Treatment Group Trials, N0234/336/436/437

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Abstract

Purpose: To investigate the associations between baseline and posttreatment circulating tumor cell (CTC) gene expression and outcome of patients enrolled in four North Central Cancer Treatment Group metastatic breast cancer (MBC) trials in which specimens were shipped (at 4°C) from community-based sites to a reference laboratory (Mayo Clinic, Rochester, MN).

Experimental Design: Blood was collected at treating sites from MBC patients before (baseline), during, and at the end of treatment with erlotinib + gemcitabine (N0234), sorafenib (N0336), irinotecan + cetuximab (N0436), or paclitaxel-poliglumex + capecitabine (N0437). CTCs from 10 mL of EDTA blood were enriched with CD45 depletion, 24 to 30 hours postblood collection. Reverse transcription/quantitative PCR was used to determine cytokeratin-19 (CK19) and mammaglobin (MGB1) mRNA levels in CTCs from up to 13 (N0234), 16 (N0336), 18 (N0436), and 39 (N0437) patients. The gene expressions were normalized to β2-microglobulin and calibrated to healthy blood using the 2−ΔΔCq algorithm; positivity was defined as 2 or more.

Results: CK19 mRNA cells were detected in 56% to 75% and MGB1 mRNA cells in 23% to 38% of 86 patients at baseline. CK19 mRNA cells were detected in 30% to 67% and MGB1 mRNA cells in 14% to 64% of 110 postbaseline serial samples. The presence of baseline CK19 mRNA cells (P = 0.01) but not MGB1 mRNA cells (P = 0.14) was significantly associated with shorter overall survival. A decrease in MGB1 mRNA levels (baseline-week 8) seemed to be associated with clinical response (P = 0.05).

Conclusions: CTC gene expression analysis conducted by a reference laboratory is feasible when blood is collected from treating sites and processed 24 to 30 hours postcollection. The presence of baseline CK19 mRNA CTCs was associated with poor prognosis; a decrease in MGB1 mRNA CTCs may help predict response to therapy of MBC patients. Clin Cancer Res; 17(22); 1–11. ©2011 AACR.

Introduction

Breast tumors shed malignant epithelial cells into the circulation, which can cause disease relapse. As metastasis is the main cause of death in patients with solid tumors, accurately detecting and characterizing these circulating tumor cells (CTC) can positively impact the management of breast cancer patients with metastatic disease (1, 2). The clinical utility of CTCs in breast cancer has shown promise in predicting risk, tailoring treatment, response monitoring, and developing novel therapies (3–5). The CellSearch Assay (Veridex) is an immunofluorescent-based cell imaging system for enumerating CTCs (6, 7) and is Food and Drug Administration (FDA) approved for metastatic breast cancer (MBC) patient prognosis and treatment monitoring. The presence and persistence of 5 or more CTCs per 7.5 mL
BreastCancerSelect (AdnaGen AG) is a multiplex PCR test for detection and treatment monitoring (12). The AdnaTest evaluation of the clinical significance of CTCs in risk prediction and treatment monitoring is becoming increasingly important in determining breast cancer patient prognosis and treatment failure (6–9) has been shown to be a superior surrogate endpoint than current radiology imaging (10) and is, perhaps, superior to standard measurements of tumor burden (11). In addition to CTC enumeration, gene expression analysis of CTCs is becoming increasingly important in determining breast cancer patient prognosis and treatment response and understanding metastatic biology. The high multiplexing capabilities associated with reverse transcription/quantitative PCR (RT-qPCR) allows for CTCs to be characterized both molecularly and biologically. Specific profiles of CTCs may result in a more effective evaluation of the clinical significance of CTCs in risk prediction and treatment monitoring (12). The AdnaTest BreastCancerSelect (AdnaGen AG) is a multiplex PCR test that detects HER2, mucin 1 (MUC1), and epithelial glycoprotein (GA733-2) transcripts (13). This test has shown clinical significance of CTCs in breast cancer patients (14), but is not FDA approved and was not available in the United States at the time of our analyses. Other multiple marker assays that include cytokeratin-19 (CK19), mammaglobin (MGB1), and HER2 are being developed to effectively detect CTCs with promising clinical significance (i.e., predicts outcome and treatment response of patients) in early- and late-stage disease (15–20). Thus, characterizing CTCs in breast cancer patients may result in an important clinical tool to aid in staging, predicting prognosis, and in designing more personalized therapeutic regimens for these patients (12).

Characterization of CTCs is attractive because blood collection is a minimally invasive procedure, and blood can be shipped from the community laboratory to the research center. However, the timing between blood collection and processing may be critical for certain analytes. Special blood collection tubes with proprietary preservatives or special phlebotomy/handling requirements are necessary for the CellSearch Assay and the Adna tests. For gene expression analyses of CTCs, EDTA tubes are typically used and in most reported studies, the blood has been processed and CTCs isolated within 2 to 4 hours after collection at the same institution of blood collection. This makes it difficult to translate a gene expression–based CTC test to local/treating sites, many of which do not have access to the required instrumentation for RT-qPCR. As such, the blood needs to be shipped overnight to a reference laboratory. Therefore, we examined whether gene expression in CTCs can still predict for outcome and treatment response for patients with metastatic breast cancer (MBC) when blood is collected from local/treating sites (stored at 4°C) and CTCs are isolated from EDTA blood 24 to 30 hours postcollection at a reference laboratory. We observed that the presence of baseline CK19+ mRNA CTCs was associated with traditional poor prognostic indicators (≥3 metastatic sites and higher grade), a trend toward shorter progression-free survival, and significantly shorter overall survival, independent of number of metastatic sites and grade. We also observed that a decrease in mammaglobin+mRNA CTCs may help predict response to therapy of MBC patients. Our data confirm literature findings and indicate for the first time that CK19 gene expression analysis in CTCs conducted by a reference laboratory still predicts for outcome of advanced breast cancer patients when blood is collected from local sites and processed 24 to 30 hours postcollection in the context of the North Central Cancer Treatment Group treatment trials. This will allow for a wider application of CTC gene expression monitoring in clinical trials and different practice settings. Validation studies are ongoing to confirm these findings.
treatment end, typically at disease progression. Although we did not prespecify the number of patients for this CTC correlative study, blood samples for CTC analysis were not collected on all patients enrolled in the clinical trials. Compliance by the local treating hospitals and clinics of blood sample collection for CTC analysis was low (22%) for the first study (N0234) in which CTC analysis was implemented in 2002. Appropriate site training improved compliance to 80% for N0336 initiated in 2003 and 85% for N0436 and N0437 initiated in 2004. A patient flow diagram describing the number of patients and blood samples (baseline and serial) for CTC analysis is provided in Supplementary Fig. S1. Comparisons of the clinicopathologic characteristics between cohort and noncohort patients with baseline and week 8 samples are provided in Supplementary Tables S2 and S3. More noncohort patients seemed to have 3 or more metastatic sites than cohort patients (P = 0.01; Supplementary Table S2).

Blood samples (~10 mL) for CTC gene expression analysis were collected in EDTA tubes and were drawn in conjunction but after routine clinical/hematologic blood draws to minimize skin epithelial cell contamination. Blood samples were collected at the local treating hospitals and clinics, stored at 4°C, and were shipped, on ice packs (4°C) overnight to the NCCTG research base, Mayo Clinic, Rochester, MN.

Between 24 and 30 hours postblood collection, CTCs were isolated from blood samples with the RosetteSep Human CD45 Depletion Cocktail followed by density centrifugation over Ficoll-Paque according to the manufacturers’ instructions (StemCell Technologies Inc.). Density centrifugation over Ficoll-Paque is necessary for the appropriate separation of rosetted red blood and unwanted cells from the mononuclear cells of interest. The enriched peripheral blood mononuclear cell (PBMC) layer was isolated and washed thrice with PBS/2% FBS. The PBMC pellet was resuspended with lysis binding buffer, and the cell homogenate was stored frozen at ~80°C until processed for mRNA isolation.

mRNA isolation, reverse transcription, and second-strand synthesis

The procedures for mRNA isolation and cDNA synthesis have been previously described (15). In brief, mRNA was isolated from the cell lysate by the mRNA Direct Kit and Dynabeads Oligo (dT)$_{25}$ according to the manufacturers’ instructions (Dynal; Invitrogen). A solid phase cDNA library was generated using AMV reverse transcriptase and oligo (dT)$_{25}$ priming and stored at 4°C. Gene-specific cDNA second-strand synthesis was carried out using gene-specific forward primers as previously described (15).

Quantitative real-time PCR

$\text{CK19}$, $\text{MBG1}$, and $\beta$-microglobulin (B2M) mRNA levels were quantified by qPCR and hydrolysis probes chemistries by a BioRad iCycler IQ. The primers and probes for $\text{CK19}$ (NM_002276.3) were designed with Universal Probe Library from Roche Applied Science. The forward 5’-GTCATGCGCGAGCAGAAC and reverse 3’-CCGTTATCA-ATTCTTAGTCC primers were obtained from Integrated DNA Technologies. The probe (GGATGCTG) was obtained from Roche Applied Science. The primers and probe for $\text{MBG1}$ (HSU33147) were designed with Primer Express from Applied Biosystems Inc. The forward 5’-AGAACCTG-CAGGGTATGGTGAA and reverse 3’-ACATTTAG-CAGCCTTCACATG primers and the probe- (6-FAM)CCTACACATGCTGCAACAGC [BHQA1-6FAM] were obtained from Integrated DNA Technologies. The primers and probe for B2M (NM_004048) were designed with Beacon Designer from PREMIER biosoft International. The forward 5’-GATTTCCTGAAGCTGACAGCATT and reverse 3’-CAGAAAAGAGAGTTAGCCGAG primers and the probe- (6-FAM) TGTCTCCTGCTCGT-GCCTTAGCTG [BHQA1-6FAM] were obtained from Integrated DNA Technologies. Specific oligonucleotides representing the respective amplicons were obtained from Integrated DNA Technologies and were used to construct standard curves for the analytes. The oligonucleotides were serially diluted in Tris-EDTA buffer and 8 to 9 working concentrations (in multiples of 10) between 10 transcript copies per 5 μL and 10$^9$ transcript copies per 5 μL for CK19 and MBG1 and between 10 transcript copies per 5 μL and 10$^9$ transcript copies per 5 μL for B2M were assayed in duplicate. The average ± SD intrarun and interrun (13 runs total for the 4 clinical trials) percent coefficient of variation (%CV) for CK19 across the oligonucleotide transcript levels were 1.58% ± 0.40% and 10.5 ± 6.44%, respectively. The average intrarun and interrun %CV for MBG1 across the oligonucleotide transcript levels were 1.30 ± 0.96 and 8.21 ± 3.59, respectively. The average (± SD) intrarun and interrun %CV for B2M across the oligonucleotide transcript levels were 0.82% ± 0.38% and 11.3% ± 6.61%, respectively. These %CVs are within the FDA guidelines that recommend %CV values of less than 15% for analytic assay precision and at least two thirds of the oligonucleotide transcript levels (standards) have %CV less than 15% (21).

Appropriate negative (e.g., water and known amount of mRNA from the MDA MB-361 cell line that contains the marker of interest reverse transcribed without AMV reverse transcriptase) and positive (e.g., specific amplicon oligonucleotides and known amount of mRNA from the MDA MB-361 cell line reverse transcribed with AMV reverse transcriptase) controls were included in each qPCR assay. In accordance to the Minimum Information for Publication of Quantitative PCR Experiments guidelines (22), the specific oligonucleotide standard curves were used to optimize amplification efficiency and to ensure that reaction efficiencies are comparable between genes of interest and the reference gene. In addition, blood samples obtained from healthy individuals were identically processed and were analyzed in each RT-qPCR run. The analytic detection limit of our model assay system is 1 in 10$^6$ cells (15), and with oligonucleotide standard curves, we can routinely detect at least 3 transcript copies per reaction. All patient samples were run in triplicate, and a sample had to have at least 2 Cq values (Cq is defined as the fractional cycle number at which
the fluorescence generated by cleavage of the probe passes a fixed threshold above baseline] less than 40 to be evaluable for the respective transcript. Samples with at least 2 Cq values of 40 were considered nondetectable. The average Cq value was used as the quantitative value, and the average %CVs (± SD) of the detectable samples were 1.66 ± 0.12, 0.97 ± 0.07, and 0.48 ± 0.02 for CK19, MGB1, and B2M, respectively, across the 4 studies.

The target messages in the CTC samples were determined using a relative quantification method (22–24). The relative quantification, 2–ΔCq algorithm, is the fold expression relative to a calibrator (i.e., blood from healthy individuals; “normal blood”) and normalized to a reference gene (B2M). For the CK19 calibrator, of 62 blood samples tested from healthy individuals, CK19 Cq less than 35 was observed in 11% of samples. For the MGB1 calibrator, of 56 samples tested from healthy individuals, MGB1 Cq less than 35 was observed in less than 2% of samples. The average calibrator ΔCq value was obtained from these samples and individual ΔCq of the calibrator were within 2 SDs of the average calibrator ΔCq value. Samples were classified as positive for a particular gene if the 2–ΔΔCq was 2.0 or more (i.e., 100% or more than what is found in healthy blood). Samples with nondetectable target messages (Cq = 40) were assigned a 2–ΔΔCq value of 0.

Statistical analysis

The primary endpoints of this analysis were to evaluate the impact of baseline and follow-up changes of CK19 mRNA+ and MGB1 mRNA+ CTCs on progression-free survival (PFS) and overall survival (OS) of MBC patients enrolled in 4 NCCTG phase II treatment trials. This study followed REMARK guidelines (25). Wilcoxon rank-sum tests were used to assess changes in CK19 and MGB1 gene expression levels between baseline and week 8 and treatment end. χ2 tests were used to determine whether CK19 and MGB1 gene expression values differed between studies. Nonstratified Cox regression models and Kaplan–Meier curves were used to determine associations between PFS and OS and CK19 mRNA and MGB1 mRNA positivity. Multivariate modeling methods included backward elimination, stepwise and assessing all potential subset models based on score criterion. The Kaplan–Meier method was used to estimate OS and PFS. Median follow-up was 2.3 years. For each clinical trial, clinical response rates as defined by Response Evaluation Criteria in Solid Tumors (RECIST) v1.0 criteria (26) among patients included in this study are listed in Supplementary Table S1.

Results

Distribution of CK-19 and MGB1 relative gene expression levels

Table 1 shows that 38% and 67% of patients had CK19+ mRNA and MGB1 mRNA levels less than 2.0, respectively, at baseline. Of the 53 patients with CK19 2–ΔΔCq of 2 or more at baseline, the relative gene expression level ranged between 2.1 and 16,286 (mean: 569 median: 14; Supplementary Table S4). Of the 28 patients with MGB1 2–ΔΔCq of 2 or more at baseline, the relative gene expression level ranged between 2.3 and 5806 (mean: 354 median: 14; Supplementary Table S5).

Incidence of CK-19mRNA± and MGB1mRNA± CTCs

CK19 mRNA+ CTCs (at 2–ΔΔCq ≥ 2) were detected in 55% to 75%, 44% to 60%, and 30% to 67% patients at baseline,
MGB1 mRNA+ CTCs were detected in 23% to 38%, 22% to 64%, and 14% to 60% patients at baseline, during, and treatment end, respectively (Table 1, Supplementary Fig. 2B). No significant differences were observed between the time points across the 4 studies. At baseline, the incidence of CK19 mRNA+ CTCs (62%; 95% CI: 51–72) was higher than the incidence of MGB1 mRNA+ CTCs (33%; 95% CI: 23–42). Of the 28 MGB1+ mRNA samples, 23 (82%) were also CK19 mRNA+ (Supplementary Table S6), and of the 53 CK19+ mRNA samples, 23 (43%) were also MGB1 mRNA+. At baseline, CK19+ mRNA and MGB1+ mRNA incidence was significantly correlated (Supplementary Table S6; \( P = 0.007 \)), and the relative gene expression levels between CK19 and MGB1 were weakly correlated (Spearman \( r^2 = 0.23; P = 0.03 \)).

Correlation between CTC positivity and clinicopathologic characteristics

The presence of CK19 mRNA+ CTCs \((2^{-\Delta\Delta Ct} \geq 2)\) at baseline correlated with hormone receptor positivity, more than 3 metastatic sites, and nonductal histology but was not correlated with nodal status (Table 2). The presence of MGB1 mRNA+ CTCs \((2^{-\Delta\Delta Ct} \geq 2)\) at baseline correlated with hormone receptor positivity (Table 2). Association between baseline CTC positivity and patient survival

Univariate analysis showed that histologic grade and performance status were independent significant predictors of PFS (Table 3). The HR for PFS was 1.37 (95% CI: 0.87–2.18; \( P = 0.18 \)) for patients with CK19 mRNA+ CTCs \((2^{-\Delta\Delta Ct} \geq 2)\) at baseline (Table 3, Fig. 1A). PFS for patients with and without MGB1 mRNA+ CTCs were
similar, and there was no significant association between baseline MGB1 mRNA+ positivity and PFS (Fig. 1B). A comparison across 4 subgroups based on baseline CTC gene expression positivity (e.g., CK19 mRNA-/MGB1 mRNA−; CK19 mRNA−/MGB1 mRNA−; CK19 mRNA+/MGB1 mRNA−; CK19 mRNA+/MGB1 mRNA+) showed no significant differences in PFS (Supplementary Table S8).

Univariate analyses showed that histologic grade, number of metastatic sites, and CK19 mRNA+ CTCs were independent predictors for OS (Table 3). Patients with CK19 mRNA+ CTCs had a significantly worse OS than patients without CK19 mRNA+ CTCs at baseline (Fig. 1C). The HR for patients with CK19 mRNA+ CTCs was 2.05 (95% CI: 1.16–3.62; Fig. 1D). The HRs for patients with CK19 mRNA+ and MGB1 mRNA+ CTCs and CK19 mRNA or MGB1 mRNA+ CTCs were 1.85 (95% CI: 1.07–3.20; P = 0.03) and 1.91 (95% CI: 1.04–3.51; P = 0.04), respectively (Fig. 1E). In a multivariate analysis, the histologic grade and number of metastatic sites were not significant covariates in the prognostic utility of CK19 mRNA+ CTCs on OS. CK19 mRNA+ (P = 0.001) and estrogen receptor negativity (P = 0.007) were the only predictors of worse OS in the final multivariate model (Supplementary Table 7).

An analysis assessing the association of baseline CTC gene expression positivity with OS showed that patients with CK19 mRNA+/MGB1 mRNA− CTCs had a nonsignificant HR of 1.77 (P = 0.10) and patients with CK19 mRNA+/MGB1 mRNA+ had a significant HR of 2.44 (P = 0.01) when compared with patients with CK19 mRNA−/MGB1 mRNA− (Supplementary Table S9).

### Association between baseline and week 8 CTC positivity change and patient OS

Patients who remained positive (ΔΔCq ≥ 2) between baseline and week 8 for CK19 mRNA+ (HR: 2.67; P = 0.05) or MGB1 mRNA+ (HR: 2.13; P = 0.09) CTCs seemed to have a worse OS than patients who remained negative (ΔΔCq < 2) for CK19 mRNA or MGB1 mRNA CTCs, respectively (Table 4). Similarly, the patients who were positive for CK19 mRNA CTCs at baseline irrespective of status at 8 weeks had a worse OS outcome than patients who were negative at baseline irrespective of status at 8 weeks (HR = 2.7; P = 0.01). The patients who were positive for MGB1 mRNA CTCs at baseline irrespective of status at 8 weeks tend to have a worse OS outcome than patients who were negative at baseline irrespective of status at 8 weeks (HR = 1.91; P = 0.07).

### Table 3. Univariate analysis for PFS and OS

<table>
<thead>
<tr>
<th>Covariate</th>
<th>HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.00 (0.98–1.02)</td>
<td>1.0</td>
</tr>
<tr>
<td>ER (negative vs. positive)</td>
<td>1.48 (0.94–2.32)</td>
<td>0.09</td>
</tr>
<tr>
<td>PgR (negative vs. positive)</td>
<td>1.27 (0.80–2.02)</td>
<td>0.32</td>
</tr>
<tr>
<td>Histologic tumor grade Elston/SBR (poor vs. well/intermediate)</td>
<td>1.89 (1.05–3.40)</td>
<td>0.03</td>
</tr>
<tr>
<td>Node (positive vs. negative)</td>
<td>0.89 (0.57–1.38)</td>
<td>0.59</td>
</tr>
<tr>
<td>Menopausal status (&lt;50 vs. ≥50 y)</td>
<td>1.12 (0.68–1.85)</td>
<td>0.65</td>
</tr>
<tr>
<td>Histology (other vs. ductal)</td>
<td>0.86 (0.40–1.83)</td>
<td>0.69</td>
</tr>
<tr>
<td>Performance status (1,2 vs. 0)</td>
<td>1.61 (1.03–2.51)</td>
<td>0.04</td>
</tr>
<tr>
<td># Metastatic sites (≥3 vs. &lt;3)</td>
<td>1.15 (0.73–1.81)</td>
<td>0.54</td>
</tr>
<tr>
<td>CK19 (≥2.0 vs. &lt;2.0)</td>
<td>1.37 (0.87–2.18)</td>
<td>0.18</td>
</tr>
<tr>
<td>MGB1 (≥2.0 vs. &lt;2.0)</td>
<td>1.05 (0.65–1.69)</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>1.00 (0.98–1.03)</td>
<td>0.87</td>
</tr>
<tr>
<td>ER (negative vs. positive)</td>
<td>1.52 (0.90–2.55)</td>
<td>0.11</td>
</tr>
<tr>
<td>PgR (negative vs. positive)</td>
<td>1.52 (0.89–2.61)</td>
<td>0.12</td>
</tr>
<tr>
<td>Histologic tumor grade Elston/SBR (poor vs. well/intermediate)</td>
<td>2.28 (1.08–4.85)</td>
<td>0.03</td>
</tr>
<tr>
<td>Node (positive vs. negative)</td>
<td>1.12 (0.67–1.88)</td>
<td>0.66</td>
</tr>
<tr>
<td>Menopausal status (&lt;50 vs. ≥50 y)</td>
<td>1.23 (0.67–2.17)</td>
<td>0.47</td>
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<tr>
<td>Histology (other vs. ductal)</td>
<td>1.48 (0.61–3.61)</td>
<td>0.38</td>
</tr>
<tr>
<td>Performance status (1,2 vs. 0)</td>
<td>1.13 (0.67–1.91)</td>
<td>0.64</td>
</tr>
<tr>
<td># Metastatic sites (≥3 vs. &lt;3)</td>
<td>2.00 (1.18–3.41)</td>
<td>0.01</td>
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<tr>
<td>CK19 mRNA (≥2.0 vs. &lt;2.0)</td>
<td>2.05 (1.16–3.62)</td>
<td>0.01</td>
</tr>
<tr>
<td>MGB1 mRNA (≥2.0 vs. &lt;2.0)</td>
<td>1.49 (0.88–2.52)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Abbreviations: SBR, Scarff-Bloom-Richardson; ER, estrogen receptor; PgR, progesterone receptor.
Association between CTC positivity and patient response

No significant correlations were observed between treatment response and CK19mRNA+ CTCs at any time point (data not shown). At week 8, 41% (17 of 42) of patients without MGB1mRNA+ CTCs had a response compared with 19% (4 of 21) of patients with MGB1mRNA+ CTCs ($P = 0.09$). At treatment end, 38% (12 of 32) of patients without MGB1mRNA+ CTCs had a response compared with 7% (1 of 15) of patients with MGB1mRNA+ CTCs ($P = 0.03$). Table 5 shows that the decrease in MGB1 mRNA levels seemed to be larger for patients who had a treatment response compared with those who did not have treatment response at week 8 ($P = 0.05$). However, it appears that MGB1 mRNA levels increased between baseline and treatment end in patients with a treatment response ($P = 0.09$). There were no significant associations between changes in CK19+mRNA levels and treatment response (data not shown).

Discussion

The detection of CTCs has been shown to predict for poor clinical outcome and treatment failure in MBC patients (6, 7). The biological characteristics (e.g., protein and mRNA expression) of CTCs may further improve predicting risk, monitoring response, and tailoring treatment strategies for individual patients with breast cancer (4, 5, 12). As CTC molecular characterization is becoming more relevant in personalized disease management of breast cancer patients, it would be beneficial to develop a gene expression–based test in which a commonly used blood collection tube (e.g., EDTA) can be easily used by local sites and the blood easily shipped to a reference laboratory and reliably tested for molecular analysis. This would potentially increase the accessibility of these types of tests to many patients, particularly in the United States. Therefore, we determined the feasibility of analyzing CK19 and MGB1 gene expression in CTCs when isolated from EDTA blood at a central
laboratory 24 to 30 hours postcollection in context of outcome of patients enrolled in NCCTG community–based treatment trials.

We observed that CK19 mRNA was detected overall in 62% of MBC patients at baseline, a slightly higher incidence than the approximately 50% incidence of patients with 5 or more CTCs typically observed by the FDA-approved CellSearch Assay (6) and 52% incidence by the AdnaTest BreastCancer (27). In 2 individual studies (N0436 and N0437) with several patients treated in the first-line setting, we did observe similar incidences of baseline CTC positivity (55%–56%) compared with literature findings.

Table 4. OS by change in CTC positivity between baseline and week 8

<table>
<thead>
<tr>
<th>CTC change</th>
<th>N = 59</th>
<th>#Events</th>
<th>HR (95% CI)</th>
<th>P</th>
<th>1 y</th>
<th>2 y</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CK19</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.0 to &lt;2.0</td>
<td>14</td>
<td>6</td>
<td>1.0 (—)</td>
<td>—</td>
<td>85.7</td>
<td>61.9a</td>
</tr>
<tr>
<td>≥2.0 to ≥2.0</td>
<td>19</td>
<td>13</td>
<td>2.67 (0.99–7.15)</td>
<td>0.05</td>
<td>73.7</td>
<td>21.6a</td>
</tr>
<tr>
<td><strong>MGB1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2.0 to &lt;2.0</td>
<td>30</td>
<td>15</td>
<td>1.0 (—)</td>
<td>—</td>
<td>80.0</td>
<td>53.8</td>
</tr>
<tr>
<td>≥2.0 to ≥2.0</td>
<td>9</td>
<td>4</td>
<td>0.92 (0.30–2.79)</td>
<td>0.88</td>
<td>76.2a</td>
<td>63.5a</td>
</tr>
</tbody>
</table>

*Less than 10 at risk.

Table 5. Gene expression level change and treatment response

<table>
<thead>
<tr>
<th>Response group</th>
<th>Change in CTC levels from baseline to week 8</th>
<th>Change in CTC levels from baseline to end of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CK19</strong></td>
<td><strong>Mean</strong></td>
<td><strong>Median</strong></td>
</tr>
<tr>
<td>Yes (N = 21)</td>
<td>−350</td>
<td>0</td>
</tr>
<tr>
<td>No (N = 38)</td>
<td>−75</td>
<td>−2</td>
</tr>
<tr>
<td><strong>Mammoglobin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (N = 21)</td>
<td>−67</td>
<td>0</td>
</tr>
<tr>
<td>No (N = 38)</td>
<td>−32</td>
<td>0</td>
</tr>
</tbody>
</table>
MGB1 mRNA has been detected in 33% to 39% of patients (28, 29), which is within the 20% to 40% range of baseline incidence we observed using our immunodepletion/qRT-PCR method. A recent comparison analysis of the CellSearch Assay, Adna Test Breast Cancer Select/Detect, and CK19/MGB1 RT-qPCR assay detected CTCs in 36%, 22%, and 26% (CK19)/54% (MGB1), respectively, in MBC patients (30). Our increased sensitivity of CK-19+ CTCs compared with previous results may be due to different patient populations (first versus second/third/fourth line), cell enrichment and RNA extraction techniques, oligonucleotide primers, and PCR approaches (standard versus real time) used throughout the various studies. These technical differences for gene expression–based tests emphasize the importance of implementing standardize approaches to isolate and characterize CTCs, similar to what has been proposed to analyze disseminated tumor cells in the bone marrow (31).

The MGB1+ mRNA incidence was lower than the incidence of CK19mRNA+ detection. Although the majority (87%) of the MGB1mRNA+ samples were also CK19mRNA+, 43% of CK19mRNA+ samples were MGB1mRNA+ and CK19 and MGB1 transcript levels were only weakly correlated. We previously observed a significant correlation between CK19 and MGB1 expression in patients with primary breast cancer (15). The presence of CK19mRNA+ CTCs at baseline was correlated with hormone receptor positivity, nonductal histology, more than 3 metastatic sites, but not with nodal status, and the presence of baseline MGB1mRNA+ CTCs was strongly associated with hormone receptor positivity. It is interesting that significant correlations between CTC gene expression (particularly for MGB1) and hormone receptor positivity were observed. Elevated MGB1 expression has been associated with clinical and biological features defining a less aggressive tumor phenotype in breast tumors (32) including steroid receptor–positive breast tumors (33). This may help explain why baseline MGB1 gene expression did not predict for reduced PFS or OS in our study. Our results are consistent with previous findings that showed a positive association between the presence of CTCs as detected by laser scanning cytometry and RT-qPCR and distant metastases (34–36). Although earlier results suggest that the incidence of CTCs is higher for node-positive than node-negative patients (34, 36, 37), the lack of correlation between CTCs and nodal status has been previously observed and indicates that 2 distinct routes exist for breast cancer metastases (38–40). Our incidence results and clinicopathologic correlations are consistent with previous findings indicating that our immunodepletion coupled with RT-qPCR approach produces reliable gene expression analysis of CTCs.

Importantly, we observed that the presence of CK19mRNA+ CTCs is associated with shorter OS and thus, correlates with poor prognosis. We also observed a potential trend toward a predictive significance of MGB1mRNA+ CTCs in that the decrease in MGB1+ transcript levels between baseline and 8 weeks seemed to correlate with patient response to treatment. Similarly, MGB1 transcripts have been recently shown to reflect the effect of therapy on adjuvant breast cancer patients (41). As MGB1 is more highly breast specific compared with CK19, which is used as a general epithelial marker, MGB1 may be a more appropriate measure of treatment response (e.g., tumor shrinkage) for breast cancer compared with CK19 (CK19 gene expression also could be confounded by its loss through epithelial–mesenchymal transition). However, increased response rates may not always result in improved PFS or OS (42). In our study, the baseline or the change in MGB1 gene expression did not significantly predict for patient PFS or OS. In addition, the detection rate of CK19+ or MGB1+ transcripts in blood samples was not shown to be associated with tumor progression in MBC patients (30). It is also interesting that a trend (P = 0.09) was observed between increased MGB1 mRNA levels (between baseline and treatment end) and treatment response. Our initial hypothesis was that a decrease in CTC gene expression would predict that a patient would respond to treatment. We did observe this for MGB1 at the first follow-up at 8 weeks but potentially the opposite was observed at treatment end. A possible explanation could be that initially, the treatment is rapidly clearing the tumor cells from the circulation and by treatment end the tumor is shrinking, releasing cells into the circulation at a greater rate than the cells being cleared from the circulation giving the appearance that an increase in CTC MGB1 gene expression at treatment end may be indicative of treatment response. However, it is important to note that the change in MGB1 gene expression between baseline and treatment end was nonsignificant and could be due to the limited sample size and various treatment regimens used between the different clinical trials.

Our findings, however, are consistent with previous results that showed an association between the presence of CK19+ CTCs and poor prognosis (16, 27, 43, 44) and show the validity of the immunodepletion/RT-qPCR method used in this study. In addition, it seems that patients who tested negative for CK19+ CTCs at baseline, irrespective of changing to positive at follow-up time points, tend to have a better outcome than patients who tested positive for CK19+ CTCs at baseline irrespective of changing to negative at follow-up time points. This is in slight contrast to the CellSearch data that showed that patients who changed from negative (<5 CTCs per 7.5mL blood) to positive (≥5 CTCs per 7.5mL blood) had worse outcome than those patients who changed from positive to negative and the outcome of those patients who turned positive was similar to those patients who remained positive for CTCs (6).

Concern does exist about the decreased gene expression levels observed in room temperature EDTA blood samples 6 hours postcollection (45). The blood samples in our studies were stored at 4°C and shipped on ice packs. This cold temperature may slow RNA degradation as our developmental work with blood from healthy individuals spiked with 1,000 cells/mL blood of MDA-MB-361 cells and stored at 4°C suggests that mRNA levels are decreased to a lesser extent when blood was maintained at 4°C (average Cq
increase of 1.63 and 1.21 for CK19 and MGB1, respectively, as observed in our hands) versus a Cq increase of 2 to 4 when EDTA blood was kept at room temperature for 24 hours (45). A Cq increase of 6.29 and 5.14, for CK19 and MGB1, respectively, was observed when blood was collected in a CellSave tube maintained at room temperature (Supplementary Fig. 3). Although RNA degradation occurs, small transcripts are still detectable by real-time PCR, a technique that is tailored to amplify small amplicon sizes.

In addition, large and small tumor cell fragments and tumor microparticles have been observed in blood from colon cancer patients using the CellSearch Assay, and these small fragments were still associated with poor clinical outcome of the patients (46). In addition, EpCam antigenicity was still present 3 days after blood collection (47). Thus, it seems that RNA may be coming from tumor cell fragments that are still clinically significant and selected cells remain viable for several days after collection. Last, our quantification method couples immunodepletion cell enrichment with RT-qPCR for which the gene expression results are calibrated to the gene expression observed in healthy blood that is identically processed (i.e., processed 24 hours postcollection) as the samples obtained from patients enrolled in NCCTG clinical trials. The negative selection has been shown to reduce background signals of HER2 and CK19 in hematopoietic cell populations to improve the specificity of RT-qPCR (48). We used normal blood as a calibrator in our quantification method to further decrease and normalize the influence of marker gene expression potentially found in hematopoietic cells as we did observe relatively elevated CK19 gene expression (e.g., Cq < 35) in 11% of normal blood samples. Molecular characterization of a single CTC by a multiplex real-time PCR also has been shown in large quantities of contaminating leukocytes (49).

As the comparison of established assay findings with clinical outcome will define the clinically relevant threshold, clinical assays are only useful when the results can be accurately correlated with clinical outcome of cancer patients and reflect the nature of the disease (12). In our study, we showed for the first time the prognostic significance of CTC gene expression analysis when blood has been processed 24 to 30 hours postcollection in context of NCCTG MBC clinical trials, suggesting that our findings are very promising for establishing gene expression–based CTC assays for clinical utility as prognostic/diagnostic tests in a community clinic setting. This will enable a broader application of CTC gene expression analyses/studies in clinical trials and different practice settings. As this study examined a limited number of patients with different treatment regimens and lines of therapy, our results should be interpreted as hypothesis generating.

Additional CTC gene expression analyses are ongoing in other NCCTG treatment trials including early (neoadjuvant and adjuvant settings) breast cancer, MBC and advanced lung cancer trials (50) to validate the feasibility of CTC gene expression testing in a community-based setting.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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