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Development of Circulating Tumor Cell-Endocrine Therapy Index in Patients with Hormone Receptor Positive Breast Cancer

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Abstract

Background—Endocrine therapy (ET) fails to induce a response in one-half of patients with hormone receptor (HR) positive metastatic breast cancer (MBC) and almost all will eventually become refractory to ET. Circulating Tumor Cells (CTC) are associated with worse prognosis in MBC patients, but enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including ET. We developed a multi-parameter CTC-Endocrine Therapy Index (CTC-ETI), which we hypothesize may predict resistance to ET in patients with HR positive MBC.

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Author Contributions: C.P. performed the experiments, developed the CTC-biomarker scale, performed CTC analyses, wrote the pilot trial protocol, assisted in developing CTC-ETI, analyzed the data as first reviewer, reviewed clinical information, prepared figures and tables and wrote the manuscript; M.C. Muñoz. performed the experiments, assisted in development of CTC-biomarker scale, analyzed data as second reviewer, cell culture, prepared figures; D.G.T. provided central staining and analyses of primary and metastatic tissues; K.A.G. designed the study, analyzed the data, and prepared figures and tables; K.M.K. analyzed the data, prepared figures and tables of the correlation between CTC and tissues; N.T. provided clinical information, revised and edited the manuscript; M.E.B. assisted in *in vitro* experiments, revised and edited the manuscript; K.A. analyzed data, prepared figures, revised and edited the manuscript; M.C. Miller helped develop the study, co-authored protocol, and edited the manuscript; D.L.B. consented and recruited patients, and provided clinical information, revised and edited the manuscript; A.F.S., N.L.H. provided patient samples, revised and edited the manuscript; J. M.R. designed the experiments, revised and edited the manuscript; M.C.C. and D.A.C. prepared the antibodies and cell line controls, and revised and edited the manuscript; D.F.H. recruited patients, provided patient samples, designed the experiments, revised and edited the manuscript, and supervised the research.

Methods—The CTC-ETI combines enumeration and CTC expression of four markers: estrogen receptor (ER), B-cell lymphoma 2 (BCL-2), Human Epidermal Growth Factor Receptor 2 (HER2), and Ki67. The CellSearch® System and reagents were used to capture CTC and measure protein expression by immunofluorescent staining on CTC.

Results—The feasibility of determining CTC-ETI was initially established *in vitro* and then in a prospective single-institution pilot study in MBC patients. CTC-ETI was successfully determined in 44/50 (88%) patients. Eighteen (41%), 9 (20%), and 17 (39%) patients had low, intermediate, and high CTC-ETI scores, respectively. Inter-observer concordance of CTC-ETI determination was 94–95% (Kappa statistic 0.90–0.91). Inter- and cell-to-cell intra-patient heterogeneity of expression of each of the CTC-markers was observed. CTC biomarker expression was discordant from both primary and metastatic tissue.

Conclusions—CTC expression of ER, BCL-2, HER2, and Ki67 can be reproducibly measured with high analytical validity using the CellSearch® System. The clinical implications of CTC-ETI, and of the heterogeneity of CTC-biomarker expression, are being evaluated in an ongoing prospective trial.

Keywords

Circulating Tumor Cells (CTC); estrogen receptor (ER); anti-human epithelial growth receptor type 2 (HER2); Ki67; BCL-2

INTRODUCTION

Endocrine therapy (ET) is routinely recommended for patients with HR positive MBC, while patients with HR negative disease are typically offered more toxic chemotherapy as first line treatment (1). However, approximately one-half of patients with HR positive MBC do not respond to ET, 15–30% of patients treated with ET experience progression within the first two to three months of therapy, and almost all patients with HR positive MBC will eventually become refractory to ET(1).

Patients with HR positive MBC who have ET-refractory disease are treated with chemotherapy, as is done for HR negative patients. Unfortunately, only clinical judgment, or a failed trial of ET, is available to identify such patients. Moreover, such empiric decisions do not permit consideration of tumor heterogeneity, which has been implicated as one of the main barriers to effective anti-cancer therapy, but the molecular tools to quantify and monitor tumor heterogeneity have been lacking (2–4). Several studies have demonstrated that ER and Human Epidermal Growth Factor Receptor type 2 (HER2) differ from the primary cancer and metastatic biopsies in 5–30% of patients with MBC (5–7). Ideally, serial real-time analysis of expression of ER and other markers associated with response or resistance to ET could be determined before or during ET to guide therapeutic decisions. However, biopsies of metastatic tissue are difficult because they are invasive, inconvenient, and associated with potential morbidity and prohibitive costs.

Circulating biomarkers represent an appealing alternative to baseline or serial tissue biopsies to predict ET resistance and to monitor evolving tumor heterogeneity. Prior studies have demonstrated that, at least when performed by the CellSearch® System (Janssen

Diagnostics, LLC, Raritan, NJ), enumeration of circulating tumor cells (CTC) is a specific, independent predictor of progression free (PFS) and overall survival (OS) in patients with MBC (8, 9). However, CTC enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including ET. Several studies have suggested that low levels of tissue expression of ER and B-cell lymphoma 2 (BCL-2), and high levels of tissue expression of HER2 and Ki67 are associated with relative resistance to ET (10–19). Indeed, the tissue-based 21 gene recurrence score, which is strongly weighted by these four markers, has been shown to be highly prognostic in patients with early stage, HR positive, breast cancer treated with ET (20, 21).

We report development of a CTC-endocrine therapy index (CTC-ETI) that takes into account the number of CTC, and CTC-expression of markers associated with ET. We hypothesize that CTC-ER and -BCL-2 expression would predict relative sensitivity, while CTC-HER2 and -Ki67 expression would predict relative resistance to ET. In this current paper, we successfully assessed the analytical validity of the CTC-ETI, which will permit us to move forward with prospective trials testing the clinical validity of this assay.

MATERIAL AND METHODS

Human Breast Cancer Cell Lines

Human breast cancer MCF-7 cells were originally from Michigan Cancer Foundation, but directly obtained from the laboratory of Dr. Stephen Ethier and were cultured in RPMI medium 1640 (Invitrogen) with 10% fetal bovine serum (FBS) and 1mM final concentration of sodium pyruvate (GIBCO 100X Sodium Pyruvate 100 mM lot 786714). BT-474, MDA-MB-231, and Sk-Br-3 cells were obtained from the laboratory of Dr. Stephen Ethier via ATCC, Dr. Shaomeng Wang via ATCC, and Dr. James Rae's laboratory, respectively and they were cultured in DMEM medium (Invitrogen) with 10% FBS. MDA-MB-231 cells were cultured in IMEM Richter's modified medium (Cellgro) supplemented with 10% FBS. For ER positive control cells, MCF-7 cells were cultured in hormone depleted condition for 2 days using DMEM (Dulbecco's Modified Eagle's Medium (Lonza) with 4.5 g/L Glucose and without L-Glutamine or Phenol Red supplemented with 10% charcoal/dextran stripped FBS (JR Scientific, Inc.) and fixed with 1X CellSave. All cell lines were confirmed by DNA fingerprinting in February, 2011 and expanded and frozen for future use.

Reagents to Phenotype CTC

The following antigen-specific fluorescent-labeled antibodies were used to characterize ER, HER2, BCL-2, and Ki67 expression on CTC: ER- α monoclonal murine ER-119.3 antibody (Ab) (Janssen Diagnostics, LLC), HER2 monoclonal murine Her81 Ab (Janssen Diagnostics, LLC), BCL-2, monoclonal murine Ab BCL-2/(100) (BD Pharmingen), Ki67 monoclonal murine B56 Ab (BD Pharmingen) (Table S1).

In vitro Experiments and Development of Positive and Negative Control Specimens

Twenty-four mL of whole blood (WB) was drawn into three 10 cc vacutainer tubes that contain a cellular fixative (CellSave Tubes, Janssen Diagnostics, LLC), from healthy volunteers after signed informed consent approved by the University of Michigan

Institutional Review Board (IRB). Cultured breast cancer cells were spiked at different concentrations into 7.5 mL aliquots of the pooled WB, and the aliquots were processed for CTC enumeration and characterization using the CXC kit (Janssen Diagnostics, LLC).

The cultured breast cancer MCF-7 and Sk-Br-3 cell lines were used as positive and negative controls, respectively, for ER staining and as negative and positive controls, respectively, for HER2 staining. The cultured breast cancer cell lines BT-474 and Sk-Br-3 were used as positive and negative controls, respectively, for BCL-2 staining. All cultured cell lines express Ki67, but heterogeneously, so MCF-7 cells were used as a control cell line for both positive and negative staining.

CTC-ETI *in vitro* Experiments

To test the CTC-Bio-Point assay for each marker and the Bio-Score calculation for different cell lines, the following cultured human breast cancer cell lines were spiked into healthy WB and processed in the CellSearch® system in a manner identical to that described below for patient samples: MCF-7 (ER+, BCL-2+, HER2-, Ki67+), Sk-Br-3 (ER-, BCL-2-, HER2 +, Ki67 +), and MDA-MB-231(ER-, BCL-2-, HER2-, and Ki67 +).

Patient Accrual, Blood Collection, and Processing

Patients with progressive MBC scheduled to start a new therapeutic regimen of any type (ET or chemotherapy or other) were enrolled onto a prospective single-institution pilot study to establish the analytical validity of the CTC-ETI assay. All patients signed informed consent approved by the University of Michigan IRB (see full protocol in supplementary material). The trial was conducted in two stages, with futility stopping rules based on unacceptable analytical failure (Fig. S1 and Table S2). Following demonstration of an acceptable rate of successful CTC-ETI analyses in the first stage, accrual was restricted to patients with HR positive MBC (stage 2). We enrolled patients until we had at least 5 patients in each group with elevated CTC (≥ 5 CTC/7.5 ml WB) (Fig. S1).

Prior to starting a new systemic treatment, all patients had ~40 ml WB drawn into 4 CellSave tubes. These tubes were pooled and divided into four different 7.5mL aliquots for CTC enumeration and characterization using the CellSearch® system.

CTC Enumeration and Characterization

CTC were isolated and enumerated using the CXC CellSearch® Kit and CellSearch® system according to manufacturer's instructions (Janssen Diagnostics LLC, Raritan NJ) and as previously described (8, 22). Three of the four fluorescent CellSearch® channels were used to distinguish CTC from WBC (DAPI, anti-cytokeratin, anti-CD45). The fourth channel was used to measure ER, BCL-2, HER2, and Ki67 expression in separate aliquots of the pooled WB specimens, using antigen-specific phycoerythrin-labeled antibodies. Positive and negative control specimens for each marker were included in each batch, as previously stated above.

After CTC enumeration of each aliquot (23), the relative expression of each biomarker was determined to be 0, 1+, 2+, or 3+ on a scale developed from the expression of that marker in

cultured human cell lines known to express each marker (0=non-detectable; 1+= low; 2+= intermediate; 3+=high) (Fig. S2), similar to HER2 staining scale reported in previous publications (24). CTC-biomarker visual phenotyping was independently performed by two operators (CP, MCM). Discordant results were reconciled by joint readings. All CTC-biomarker results were determined without knowledge of tissue-expression of the marker, except that all of the last 40 patients enrolled were known to have had ER positive breast cancer (primary or metastatic).

Calculation of CTC-ETI

To generate CTC-ETI, assumptions were made about the relative prognostic and predictive effects of each of the biomarkers, including the use of a CTC cutpoint of ≤ 5 CTC/7.5 ml WB, based on published literature (8, 10–13, 15–19). CTC-ETI was considered low if a patient had <5 CTC/7.5 ml WB. For those patients with ≤ 5 CTC/7.5 ml WB, the percent of cells that had each category (0, 1+, 2+, and 3+) of relative expression of ER, BCL-2, HER2, and Ki67 of the CTC was used to derive the CTC-ETI. The expression was evaluated visually with the semi-quantitative scale described above. Staining of 2+ and 3+ was considered “positive”, and staining of 0 and 1+ was considered “negative”.

CTC-Enumeration Points—CTC levels were enumerated in each of the four different aliquots of 7.5mL of WB. The average CTC count of the four tubes was used to assign the CTC-Enumeration Points for that blood draw, as follows: 0 points = average <5 CTC/7.5 ml WB (favorable outcome), 1 point = average 5–10 CTC/7.5 ml WB (intermediate outcome), and 2 points = average >10 CTC/7.5 ml WB (worst outcome) (Table S3A). If the average of CTC counts was <5 CTC/7.5 ml WB, but two aliquots had ≤ 5 CTC/7.5 ml WB, the average was rounded down to the closest integer.

CTC-Bio-Points and Bio-Score—If the average CTC/aliquot was ≤ 5 /7.5mL WB, CTC-Bio-Points were determined for each marker, based on the percentage of CTC that were positive (2+ or 3+) for the respective marker. Arbitrarily, we established three categories of positive staining: 0%, 1–10%, and $>10\%$ of CTC staining for each marker (Table S3B). Since ER has an absolute role in ET response, ER expression was weighted more heavily than the other three markers. Positive CTC-ER and BCL-2 readings were given low points (= sensitivity to ET), while positive CTC-HER2 and Ki67 readings were given high points (=resistance to ET) (Table S3B). The sum of Assigned CTC-Bio-Points for each marker produces a final CTC-Bio-Score.

CTC-ETI—The CTC-Enumeration Points were combined with the CTC-Bio-Score to derive the final CTC-ETI Score (Table S3C), according to the following equations:

$$\text{CTC- ETI} = [\text{CTC- Enumeration Points}] + [\text{Bio- Points for ER} + \text{Bio- Points for BCL- 2} + \text{Bio- Points for HER2} + \text{Bio- Points for Ki67}]$$

$$\text{CTC- ETI} = [\text{CTC- Enumeration Points}] + [\text{Bio- Score}]$$

Thus, CTC-ETI can range from 0–14 (Table S3D). To make the CTC-ETI Score clinically applicable, the scores were placed into three categories, much as histologic grading is

calculated: Low CTC-ETI Score = 0–3, Intermediate CTC-ETI Score = 4–6, High CTC-ETI Score = 7–14.

No CTC-enumeration, biomarker, or CTC-ETI results were returned to the patient or her caregiver.

Tissue: Collection and Central Staining

Patient's blocks were obtained from the University of Michigan Department of Pathology archives or from the referring institution. All pathologic analysis was performed by a pathologist (DGT).

Immunohistochemical Staining of Breast Cancer FFPE Specimens

Immunohistochemical staining was performed on a DAKO Autostainer (DAKO, Carpinteria, CA) using diaminobenzadine (DAB) as the chromogen and the detection system as noted below. De-paraffinized sections of formalin fixed tissue at five-micron thickness were stained with ER, BCL-2, HER2, and Ki67 antibodies (Table S1). Appropriate negative (no primary antibody) and positive controls (breast carcinoma) were stained in parallel with each set of tumors studied.

ER- α , BCL-2, and HER2 were scored using the Allred system(25). Ki67 slides were scanned into an APERIO imaging system and the percent positive cells calculated using the positive nuclear algorithm. ER and BCL-2 were considered positive if Allred score was ≥ 3 , and HER2 was considered positive if Allred score was ≥ 4 . For Ki-67, 10% cells staining was considered positive.

Statistical Analysis

Study design—Feasibility and analytical validity of determination of CTC-ETI was designed by using two-step futility clinical trial design (see Fig. S1 and Supplementary Material for full protocol, including planned statistical analysis). Success of the CTC-ETI assay was defined as the ability to measure each of the phenotypic markers of the ETI (ER, BCL-2, HER2, and Ki67) on $\geq 60\%$ of CTC-informative patients (those patients with an average of ≥ 5 identified CTC/7.5 ml of WB). Initial accrual goal was 20–40 patients, depending on successful determination of CTC-ETI in each stage. The trial was amended to accrue up to 50 patients for additional experience.

This study is reported according to the REMARK guidelines (26).

Reproducibility of CTC-ETI

CTC Enumeration: The average number and coefficient of variation (CV: standard deviation/mean) of CTC enumeration was calculated for each of four possible aliquots per patient.

Inter-reviewer Reproducibility of CTC-ETI: Concordance of CTC-ETI between reviewers was assessed using the Kappa agreement statistic and 95% confidence interval. The statistic was calculated across all CTC with identified staining levels for each marker.

Concordance between CTC and tissue biomarker expression. Spearman rank correlation and Kappa coefficients were determined for agreement between tissue and CTC expression of each biomarker (25). To calculate the kappa coefficient, we dichotomized the Allred and visual scores between positive and negative by using the cutoff for each biomarker as described above. For CTC, 10% of cells staining of 2+ or 3+ was considered positive.

RESULTS

Preclinical development of the CTC-ETI assay

The overall goal of this set of investigations was to develop a CTC-based biomarker index for patients with HR positive MBC starting a new ET. Prior to initiation of any clinical studies, we performed pre-clinical studies to develop the technical aspects of the CTC-ETI assay.

Characterization of antibodies in cultured breast cancer cell lines—As illustrated in Fig. S3A–D, each antibody appropriately stained positive or negative for the respective marker in the selected cell lines. To model what we anticipated in the subsequent clinical studies, we developed a semi-quantitative immunofluorescent staining scale (0, 1+, 2+, and 3+) determined by visual readings for each biomarker against the respective cell lines (Fig. S2). The location and degree of staining for each marker in these cell lines was then used in subsequent batches as internal positive and negative controls.

Calculation of CTC-Bio-Points and CTC-Bio-Score—As proof of principle, we calculated a CTC-Bio-Score (Table S3), for the cultured human breast cancer cell lines known to represent various intrinsic subtypes (MCF-7= luminal, Sk-Br-3= HER2-like, and MDA-MB-231= basal). As expected, the luminal-type, hormone-depleted MCF-7 cells were assigned 0 Bio-Points for CTC-ER and CTC-BCL-2, since in both cases >10% of the cells stained either 2+ or 3+ (Fig. S4A). Further, MCF-7 cells were assigned a CTC-HER2 Bio-Point of 1, since 1% of the cells stained 2+ for this biomarker, and CTC-Ki67 Bio-Point of 2, since more than 10% of the cells stained 2+–3+ (Fig. S4A). In contrast, the basal-like MDA-MB-231 cell line was assigned 6 CTC-ER Bio-Points, since 0% of the cells were 2+ or 3+ for this biomarker. This cell line was also assigned 1, 0, 2 Bio-Points for BCL-2, HER2, and Ki67, respectively (Fig. S4B). The HER2-amplified Sk-Br-3 cell line was assigned 6, 2, 2 and 2 Bio-Points for ER, BCL-2, HER2, and Ki67, respectively (Fig. S4C).

For each cell line, a CTC-Bio-Score was then calculated by summing the Bio-Points assigned to it for each of the biomarkers, as explained in Table S3. As expected, the MCF-7 cell line had a relatively low CTC-Bio-Score (=3), while MDA-MB-231 and Sk-Br-3 cell lines had higher CTC-Bio-Scores (= 12 and 9, respectively) (Fig. S4, Table S4).

Heterogeneity of cell line CTC-biomarker expression—Even though these experiments were performed using cultured human breast cancer cell lines grown and harvested under optimal conditions, we observed remarkable intra-line heterogeneity of biomarker staining as illustrated for ER staining of hormone depleted MCF-7 cells (Fig. S5). Two-thirds of the cells stained 2+, while 18% and 15% stained 1+ or 0, respectively. Similar heterogeneity was seen for BCL-2, HER2, and even Ki67. Ki67 expression for each cell line

was consistently >10%, as expected, but, not all cells were positive for this proliferative marker within any of the cell lines.

CTC-ETI in Patients with Metastatic Breast Cancer

CTC-ETI in pilot clinical trial—Following pre-clinical development of the CTC-Bio-Point/Bio-Score assays, we extended the CTC-ETI assay to patients with MBC (Fig. S1 and Figure 1). CTC ETI was successfully determined in all five of informative patients (of eight total) patients enrolled onto the first stage of the protocol. One patient in Stage 2 Group 1 was ineligible because she had ER negative breast cancer and was replaced with a new patient. CTC-ETI was successfully determined for three of the five informative (5 CTC/7.5 ml WB) patients enrolled into Group 1, two of the five informative patients in Group 2, and four of five informative patients in Group 3. In summary, in Groups 1–3, CTC was determined successfully in 9 of 15 informative patients, exceeding our protocol-stipulated criterion for successful analytical validity (9 of 15 informative patients). Examples of CTC-ETI calculation from three selected patients who had low, intermediate, or high CTC-ETI are provided in Table S5.

CTC-ETI could not be calculated due to technical failure of the machine for one or more of the four aliquots in two patients (#15 and #20) and due to analytical failure in four patients (#10, #17, #19, and #29). Analytical failure was defined in these cases if the average enumeration of all four aliquots was 5CTC/7.5 ml WB, one of the four aliquots had <5CTC/7.5 ml WB while CTC were 5/7.5 ml WB in the other three aliquots from the same blood draw.

In order to gain additional experience with clinical determination of CTC-ETI, the protocol was amended to enroll a total of 50 patients (those included in the original feasibility plus the expansion cohort) (Figure 1). In total, CTC-ETI was successfully determined in 44 of the 50 eligible patients, ranging from 0 to 14 (Figure 1). CTC-ETI was considered low (score ranged 0–3) in 18 (41%) of the 44 patients due to either a patient having < 5 CTC/7.5 ml WB (CTC-Enumeration Points = 0)(n=15) or 5 CTC/7.5 ml WB (CTC-Enumeration Points = 1 or 2), but with low Bio-Scores (n=3) (See Table S3A–C for definitions). Nine (20%) and 17 (39%) patients had intermediate (score range: 4–6) and high CTC-ETI (score range: 7–14), respectively (Figure 1). CTC-ETI details for all patients are provided in Table S6.

Since this study was a feasibility study designed to determine analytical validity only, patients were treated with many different types of therapies (both endocrine and chemotherapy), did not necessarily have measurable disease, and outcomes were not uniformly determined. These multiple confounding issues precluded a valid exploration of associations between CTC-ETI and clinical outcomes.

Performance Characteristics of CTC-ETI

Co-efficient of variation of CTC-enumeration—The coefficient of variation (CV) for CTC-enumeration varied from 0 to 0.7% and did not vary as the mean increased (Fig. S6). As expected, the variability was higher with low number of CTC and this generally lessened with higher numbers. However, an aberrant variation was observed around 50 cells.

Inter-reader concordance of CTC-Bio-Scores—There was strong agreement in assessment of CTC-Bio-Score and CTC-ETI between the two independent reviewers, with a Kappa statistic for inter-reader concordance of > 0.9 for all of the markers (Table 1). In particular, discordances for each marker between readers that would have changed the CTC-ETI category occurred in < 3% of patients, demonstrating that the assay has high analytical reproducibility.

Taken together, these data demonstrate the robust analytical validity and inter-reviewer reproducibility of the CTC-ETI assay, as well as the broad distribution of CTC-ETI across the population of patients with HR receptor positive MBC.

Heterogeneity of CTC-Biomarkers

As expected, CTC-enumeration varied widely among the patients, ranging from 0 to 837 CTC/7.5 ml WB, as did CTC-biomarker expression. Further, we observed enormous intra-patient heterogeneity of each CTC-biomarker (Fig. 2A–D). Interestingly, even though all of these patients had ER positive breast cancer as determined by their primary institutional pathology laboratory at some point prior to study entry, 13 of the 29 evaluable patients with 5CTC/7.5 ml (45%) had negative (0–1+) CTC-ER expression (Fig. 2A). Among patients who had elevated CTC (> 5 CTC/7.5 ml WB) and some level (1, 2 or 3+) of CTC-ER expression, the relative CTC-ER expression was quite heterogeneous. CTC were not 100% positive (2+, 3+) for ER in any patient (Figure 2A). The results of patient # 4 are particularly illustrative (Fig. 2A and Fig. S7). Her primary cancer was originally 95% positive with strong staining for ER (Fig. S7). She had 790 CTC/7.5 ml WB in the aliquot evaluated for ER expression (Figure 2A). Of these, 36% were CTCER 0, 11% were 1+, 50% were 2+, and 3% were 3+.

Likewise, for most patients, intra-patient CTC-BCL-2, HER2, and Ki67 expression were highly heterogeneous (Fig. 2B–D). For example, patient #28 had metastatic tissue that was FISH negative for HER2, but CTC-HER2 staining was 0, 1+, 2+, and 3+ in 7%, 29%, 50% and 14%, respectively (Fig. 2B).

Comparison of CTC-Biomarker Expression to Cancer Tissue Biomarker Expression

We investigated the correlation between CTC and cancer tissue biomarker expression. None of the available tissues was collected at the time of the CTC draw. Each was either the excised primary cancer or a biopsy of the first metastases. Therefore, we hypothesized that CTC and tissue biomarker expression would be discordant due to natural and therapeutic-induced genetic and phenotypic drift over time.

We included patients with CTC > 1/7.5 ml WB and for whom archived breast cancer tissue was available. We excluded patients progressing on fulvestrant immediately before being enrolled in this pilot study since they were expected to have artificially induced CTC-ER negative results (n=5). Thus, 26 and 19 patients with available primary or metastatic tissues, respectively, met these criteria (Fig. 3A, B).

As expected, substantial discordance was found between CTC and cancer tissue biomarker expression, including both primary and metastatic sites (Fig. 4A–H, Table 2A–D and Table

S7). Kappa scores for CTC and tissue concordance were quite low for all the markers (Table S7). For example, of the 19 patients for whom primary cancer was available and was positive by central ER staining, 9 (47%) had negative CTC-ER (defined as <10% CTC with 2+ or 3+ ER staining) (Table 2A). Likewise, of the 11 patients who were not taking fulvestrant and for whom metastatic tissue was available and ER was positive, 7 (60%) had negative CTC-ER (Fig. 4A, Table 2A). Two of the four patients (50%) who were found to have ER negative metastatic tissue upon central staining had at least 10% of their CTC stain positively for ER (Fig. 4B, Table 2A).

Similar discordance between CTC and tissue expression was seen for the other biomarkers (Fig. 4C–H, Table 2B–D). In order to generate a more dynamic range of values than would be available using assays suggested by the American Society of Clinical Oncology and College of American Pathologists (ASCO-CAP) Practice Guidelines Committee, HER2 was determined by Allred score (25, 27). Of 10 patients with primary and nine patients with metastatic HER2 positive Allred scores, five (50%) and six (66%), respectively, had negative CTC-HER2 expression (Fig. 4D, E, Table 2C). Perhaps more importantly, of 10 and six patients with negative HER2 primary or metastatic tissues, two (20%) and four (66%) had at least 10% of their CTC stain positively for HER2.

DISCUSSION

In this study, we have established the analytical validity for determining CTC-ETI, based on immunomagnetic capture, enumeration, and biomarker characterization using the CellSearch® system in patients with HR positive MBC. We have demonstrated that CTC-ETI, and its individual components, can be reliably and reproducibly evaluated by two separate operators. The analytical failure rate (12%) was well within our protocol-stipulated acceptable limits for the assay (protocol in Supplementary Material).

Furthermore, we have observed an expected broad distribution of CTC-ETI scores across 44 patients whose primary and/or metastatic tumors were known to be HR positive. Roughly 2/3 of these patients with HR positive MBC had low or intermediate CTC-ETI scores, whereas 1/3 of these patients had high CTC-ETI scores.

We hypothesize that those patients with high CTC-ETI score are likely to be refractory to, and unlikely to benefit from, ET. Therefore, they might be better palliated with chemotherapy, in spite of the higher side effect profile of the latter. However, this pilot study was designed to determine analytical, not clinical validity (28). Entry criteria, patient characteristics, and treatments were quite broad, and therefore we did not attempt to evaluate associations between CTC-ETI and patient outcomes. Moreover, the CTC-ETI was based on a series of informed, but relatively arbitrary assumptions. These included the selection of thresholds for CTC levels and expression of each biomarker to calculate CTC-Points and Bio-Scores. In our assay, we have used external cultured human breast cancer cells that are known not to express the relative marker as negative controls for each run. Other investigators have reported using leukocytes that are still present in the assay as an internal negative control for HER2(29). At present, it is unclear which of these methods is preferable.

The intra-patient, cell-to-cell heterogeneity we observed in CTC-biomarker expression underscores our belief that the CTC-ETI algorithm will need to be adjusted according to correlation to clinical outcomes of patients with HR positive MBC. Taking these considerations together, our results are the basis of two currently accruing, multi-national, multi-institutional prospective trials specifically designed to evaluate the clinical validity of CTC-ETI: 1) the Characterization of Circulating Tumor Cells (CTC) From patients With Metastatic Breast Cancer Using the CTC-Endocrine Therapy Index, COMETI P2, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01701050) Identifier: NCT01701050; and 2) SWOG S1222, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02137837) Identifier: NCT02137837). The data generated in these trials will permit us to determine if CTC-ETI is correlated with outcomes in patients with HR positive MBC starting a new second or third line ET. They will also permit us to re-train the multi-parameter ETI algorithm to specifically identify the 15–30% of such patients who rapidly progress within the first two to three months after initiation of ET.

We and others have previously reported evaluation of various biomarkers on CTC, using CellSearch® or other systems (30). However, few if any reports have examined combining several markers into a single index designed to address a specific clinical use (in this case, resistance to ET) using rigorous analytical methodologies. The observed heterogeneity of each CTC marker highlights the complexity of such an assay. It is essential that such analytical validity be established before any tumor biomarker test can be evaluated for clinical utility (28). We have demonstrated that the CTC-ETI, based on the CellSearch® system, is highly reproducible, with strong inter-observer concordance. Indeed, our data further support a recently reported study that determined highly concordant inter-reader reproducibility of CTC analysis using the CellSearch® system (31).

Our results also further highlight the long-recognized degree of tumor heterogeneity in MBC, which is presumably the major obstacle to cure of patients with metastatic cancer (2–4, 32). Ideally, one would like to measure tumor heterogeneity in all metastatic sites, at baseline and then serially during treatment, but such an approach is costly and logistically difficult. Serial evaluation of CTC biomarker expression might provide an opportunity to monitor evolving tumor heterogeneity, especially during treatment (33). Moreover, CTC-biomarker expression, and in particular CTC-ETI, provides an integrated picture of tumor heterogeneity within the entire patient, as opposed to biopsies of individual sites (34), and serial CTC analysis may provide an opportunity to monitor the effects of treatment on CTC-biomarker expression (35). However, in the current study, we did not address whether CTC-ETI, or CTC-individual biomarker scores, changes over time in individual patients, either independently or under selective pressure of systemic therapies. This issue is also a key secondary objective of the ongoing COMETI and S1222 trials.

Several studies have demonstrated discordance in tissue biomarker expression, particularly ER and HER2, between primary and metastatic cancers (5–7, 36–40). These changes may have clinical importance in regards to ET and anti-HER2 therapy. In this regard, differences between CTC and primary tumor biomarker expression have been reported. For example, Babayan *et al.* observed that over two-thirds of women with ER positive MBC exhibited heterogeneous CTC-ER expression, although 19% were homogeneously negative (41). Similarly, we also observed discordance between CTC and tissue expression for each

biomarker. For example, one-third of our patients, all of whom were considered to have HR positive primary or metastatic cancer as determined at their local institutions, had ER negative CTC.

The appearance of HER2 positive CTC in patients whose primary or metastatic tissues were previously considered HER2 negative has been reported previously (24, 42, 43), and could broaden the indication for anti-HER2 therapies. The heterogeneity we observed in CTC-HER2 is consistent with reports demonstrating changes in the same patient in biomarker expression between primary and metastatic tumor (44), between different metastatic lesions, and even within the same tumor (45, 46). These data are also supported by studies from several investigators demonstrating up-regulation of HER2 when ER positive breast cancer cells are placed in low-estrogen environments (45, 47).

In summary, we have developed a highly analytically validated assay to provide semi-quantitative analyses of several important biomarkers combined in a single test: CTC enumeration, and CTCER, BCL-2, HER2, and Ki67 expression. The CTC-ETI assay may serve as a real-time predictive factor for resistance to ET in patients with HR positive MBC. Furthermore, serial monitoring of CTC-ETI may provide insight into mechanisms of resistance to all, or specific types of ET. The CTC-ETI data demonstrate the extensive tumor heterogeneity that exists within patients with HR positive MBC. This heterogeneity creates enormous challenges to treatments with ET and to development of new, targeted therapies. It is possible that serial CTC-biomarker evaluation will provide a pharmacodynamic tool to monitor biomarker expression over time, and help guide personalized therapeutic management for patient with metastatic malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCL-2	B-cell lymphoma
CTC	Circulating Tumor Cells
CTC-ETI	Circulating Tumor Cells Endocrine Therapy Index
CV	coefficient of variation
ET	Endocrine Therapy
ER	Estrogen Receptor

HER2	Human Epidermal Growth Factor Receptor type 2
HR	Hormone Receptor
IHC	Immunohistochemistry

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Statement of translational relevance

Circulating Tumor Cells (CTC) are associated with worse prognosis in metastatic breast cancer (MBC) patients, but enumeration alone is insufficient to predict the absolute odds of benefit from any therapy, including endocrine therapy (ET). Furthermore, CTC represent an appealing alternative to baseline or serial tissue biopsies to predict ET resistance and to monitor evolving tumor heterogeneity. We developed a multi-parameter CTC-Endocrine Therapy Index (CTC-ETI), which is based on CTC enumeration, as well as highly-validated semi-quantitative analyses of several important biomarkers (CTC-ER, BCL-2, HER2, and Ki67 expression). Our data demonstrate extensive CTC-biomarker heterogeneity in patients with hormone receptor (HR) positive MBC. This heterogeneity creates enormous challenges to treatments with ET and to development of new, targeted therapies. The CTC-ETI assay, which was analytically validated in this study, may serve as a real-time predictive factor for resistance to ET in patients with HR positive MBC.

Fig. 1.
REMARK diagram for patient enrollment and distribution.

Fig. 2.

CTC enumeration and biomarker staining for patients with HR positive MBC for the four biomarkers.

A: ER; **B:** BCL-2; **C:** HER2; **D:** Ki67. In each, the top panel demonstrates CTC/7.5mL WB on a logarithmic scale for each patient, in descending order based on CTC number. The lower panel provides the percentage of CTC that stained 0 (yellow), 1+ (fuchsia), 2+ (blue), or 3+ (green). See Fig. S2 for standard control staining of cultured breast cancer cell lines for definitions of each category. For each patient, CTC

levels varied slightly between among each aliquot marker. The screen failure patient is not included. Data from aliquots that were technical or analytical failures are not included, but the data from the remaining aliquots for the other biomarkers for that patient are included for analyses. The CTC enumeration on Y-axis is a logarithmic scale. Thus, patients with a single CTC/7.5 ml WB appear as 0.

A: Primary cancers

B: Metastatic cancers[#]

[#]One specimen available for ER and HER2 was not available for BCL-2 and Ki67.

Fig. 3.
REMARK diagrams of tissue procurement and staining
A: Primary cancers; **B:** Metastatic cancers.

Fig. 4.

Correlation of CTC and tissue biomarker expression.

A, B: ER; C, D: BCL-2; E, F: HER2; G, H: Ki67.

A, C, E, G: CTC-biomarker compared to primary cancer tissue biomarker. **B, D, F, H:** CTC-biomarker compared to metastatic cancer tissue biomarker.

For each biomarker, ● = 1–4 CTC/7.5 ml WB; ◐ = 5–10 CTC/7.5 ml WB; ◑ > 10 CTC/7.5 ml WB and relative biomarker staining score (0, 1, 2, and 3+). See Methods for details.

Allred Score: see Methods; for Ki67, results are expressed as % positive cells. Dotted lines represent cutoff levels for CTC-biomarker and for Allred score or % positive Ki67.

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Table 1

Inter-reader concordance of CTC-ETI

Marker	Concordance	Kappa (95% Conf. Limits)	CTC-ETI important discordance ^a
ER	95%	0.91 (0.90–0.92)	3%
BCL-2	94%	0.91 (0.89–0.92)	2%
HER2	95%	0.90 (0.88–0.91)	2%
Ki67	95%	0.92 (0.91–0.93)	2%

^a“Important Discordance”= would have changed CTC-ETI category for patient; Final CTC-ETI was calculated after resolution of discordance between observers

Table 2

Concordance between CTC and tissue biomarker expression

	Primary tissue			Metastatic tissue		
	Positive ^a	Negative	Total	Positive	Negative	Total
ER						
CTC	Positive ^b	10	0	10	4	2 6
	Negative	9	0	9	7	2 9
	Total	19	0	19	11	4 15
BCL-2						
CTC	Positive	12	1	13	8	2 10
	Negative	3	1	4	1	2 3
	Total	15	2	17	9	4 13
HER2						
CTC	Positive	5	2	7	3	4 7
	Negative	5	8	13	6	2 8
	Total	10	10	20	9	6 15
Ki67						
CTC	Positive	4	14	18	6	8 14
	Negative	0	2	2	1	1 2
	Total	4	16	20	7	9 16

^aFor tissue ER and BCL-2, positive = Allred Score 3; for HER2, positive = Allred score 4; for Ki67, positive = 10% staining^bFor CTC-ER, BCL-2, HER2, and Ki67, positive = 10% staining 2-3+ in sample with 1 CTC/7.5 ml of WB