Circulating Tumor Cell Analysis in Metastatic Triple-Negative Breast Cancers


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Introduction

The detection of rare circulating tumor cells (CTC) in the blood of patients with cancer is extremely challenging. Several CTC enumeration studies using the FDA-cleared CellSearch (Veridex) have demonstrated the predictive and prognostic value of CTCs in metastatic breast cancer (1–9). Patients with CTCs at or above the threshold of 5 per 7.5 mL blood before treatment and those who fail to clear the cells during treatment have a significantly worse outcome than those who maintain a CTC count <5 after starting systemic therapy (2). A recent randomized phase III trial found that enumerating CTCs using the commercially available CellSearch assay to support treatment decisions did not lead to improved outcomes (10). Moreover, most commercial assays use methodologies that cannot capture viable cells, limiting their utility in correlative science analyses and in the emerging use of blood-based assays to identify resistance mechanisms (11). Novel CTC detection assays have been developed that can be used for genomic and other molecular profiling (11, 12). Whether these newer methods identify the same CTC population and provide similar clinical implications as the CellSearch system has not been evaluated.

The CellSearch system involves a two-step process, initial EPCAM-based immunomagnetic enrichment is followed by immunofluorescence microscopy. Here, CTCs are defined as nucleated cells, which express cytokeratin but do not express the leukocyte-specific marker, CD45. Our group has developed a similar enumeration method, referred to as IE/FC, which also involves an EPCAM-based immunomagnetic enrichment (IE) step. However, instead of microscopy, our method uses flow cytometry (FC) analysis and maintains cell viability (13). During flow cytometry, events that are positive for EPCAM and a nuclear marker, CD45, are scored.

Purpose: Recent developments in rare-cell technology have led to improved blood-based assays that allow for the reliable detection, enumeration, and more recently, genomic profiling of circulating tumor cells (CTC). We evaluated two different approaches for enumeration of CTCs in a prospective therapeutic study of patients with metastatic triple-negative breast cancer (TNBC).

Experimental Design: The CellSearch system, a commercially available and U.S. Food and Drug Administration (FDA)—cleared assay for CTC enumeration, and IE/FC, an alternative method using EPCAM-based immunomagnetic enrichment and flow cytometry that maintains cell viability, were used to enumerate CTCs in the blood of patients with metastatic TNBC. CTC numbers were assessed at baseline and 7 to 14 days after initiation of therapy with cetuximab ± carboplatin in a phase II multicenter clinical trial (TBCRC 001).

Results: CTC numbers from two methods were significantly correlated at baseline (r = 0.62) and at 7 to 14 days (r = 0.53). Baseline CTCs showed no association with time-to-progression (TTP), whereas CTCs at 7 to 14 days were significantly correlated with TTP (CellSearch P = 0.02; IE/FC P = 0.03). CTCs at both time points were significantly associated with overall survival (OS) [CellSearch: baseline (P = 0.0001) and 7 to 14 days (P < 0.0001); IE/FC: baseline (P = 0.0009) and 7 to 14 days (P = 0.008)]. Conclusions: Our findings demonstrate that CTC enumeration by two different assays was highly concordant. In addition, results of both assays were significantly correlated with TTP and OS in patients with TNBC. The IE/FC method is also easily adapted to isolation of pure populations of CTCs for genomic profiling.
CTC enumeration with the CellSearch system

Blood samples were collected into CellSave tubes (Veridex, LLC) and processed within 96 hours after blood draw. A total of 7.5 mL of blood was subjected to CellSearch enumeration using the CellSearch Circulating Tumor Cell Kit (Veridex, LLC) following the manufacturer’s instructions without modifications (15). In brief, tumor cells were immunomagnetically captured away from peripheral blood cells using iron beads coated with anti-EPCAM monoclonal antibody (mAb) and then identified by fluorescence microscopy using the following definition: cytokeratin-positive, CD45-negative, and nucleated.

CTC enumeration with IE/FC

Blood samples were collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Approximately 10 mL of blood was subjected to IE/FC as previously described (11, 13, 16–18). All IE/FC samples were processed within 36 hours. Briefly, immunomagnetic particles coated with EPCAM (MI37) mAb and another EPCAM (EB-1) mAb conjugated to phycoerythrin (PE) were added to whole blood. The sample was then subjected to a magnetic field, and unbound cells, including red blood cells, were aspirated. The bound cells were then subjected to another round of magnetic separation. The cells were resuspended in 150 μL of casein buffer and were stained with 20 μL of a 1:1 mixture of a proprietary nucleic acid dye (BD) and a leukocyte-specific CD45 (2D1) mAb conjugated to peridinin-chlorophyll-protein-Cy5.5. Next, 350 μL of phosphate-buffered saline (PBS) was added to the sample. The total volume (~520 μL) was then transferred to a TruCount tube before flow cytometric analysis using the FACSCalibur (BD Biosciences). In initial validation studies, the IE/FC assay was highly reproducible, and exhibited high correlation with the standardized CellSearch assay on shared test samples prepared using spiked cancer cells.

Statistical analysis

Enumeration results for IE/FC were reported as CTCs per mL of blood (CTC/mL) while CellSearch results were reported as number of CTCs per 7.5 mL of blood or as CTC/mL (Table 1). The TBCRC 001 clinical trial showed no difference in overall survival (OS) between the two treatment arms (14), therefore, CTC results from patients in each arm were combined. Also, the analysis was limited by the relatively low number of events. The primary goals of this study were (i) to evaluate the concordance between the two CTC enumeration methods (CellSearch vs. IE/FC); and (ii) to determine association of CTC numbers with time-to-progression (TTP; defined from treatment initiation to documented progression) and OS (defined as time from treatment initiation to death due to any cause). The Pearson correlation coefficient and Spearman rank correlation tests were used to evaluate the correlation of CTC numbers between the two methods, and P < 0.05 was considered statistically significant. In addition, kappa statistics was calculated to determine the degree of concordance of CTC cutoffs. TTP and OS were estimated using the Kaplan–Meier method and the log-rank test was used to compare TTP and OS between groups, with P < 0.05 being considered statistically significant. Two cutoff values were used for analysis: ≥0.67 CTC/mL [i.e., ≥5 CTCs/7.5 mL], the standard CellSearch cutoff;
ref. 2, 3), and ≥1 CTC/mL. No prognostic significance was observed when patients were dichotomized on the basis of detectable CTCs versus those without. Cox proportional hazard ratios with 95% confidence interval (95% CI) were calculated to estimate risk of progression or death using multivariate Cox regression models for dichotomized groups from the two CTC enumeration methods.

**Results**

**Patients**

The TBCRC.001 clinical trial was a two-arm randomized phase II study of the EGFR antibody cetuximab with or without carboplatin in patients with metastatic TNBC. Baseline clinical and demographic data of the 102-patient cohort were previously described in detail (14). The median follow-up was 26 months, during which 85 of the 102 patients experienced progression, and 75 of whom died (14).

**Enumeration of CTCs**

Blood samples were obtained from all 102 metastatic TNBC patients at pretreatment (baseline) and 7 to 14 days after the initiation of therapy using the CellSearch and IE/FC methods (Table 1 and Supplementary Table S1). CellSearch values were expressed as CTC/mL to conform with the IE/FC readout, and also as CTCs/7.5mL to reflect the standard CellSearch readout.

**CellSearch.** Using CellSearch, CTCs were successfully enumerated in blood samples from 95 and 89 patients at baseline and at 7 to 14 days after initiation of therapy, respectively (Table 1 and Supplementary Table S1). Results were not available from 7 samples at baseline and 13 samples at 7 to 14 days due to several reasons, including insufficient blood volume, clotted blood samples, and instrument failure (Supplementary Fig. S1). Forty-two (44%) and 29 (33%) patients had ≥5 CTCs/7.5mL of blood at baseline and 7 to 14 days, respectively (Table 1).

**IE/FC.** We enumerated CTCs using the IE/FC method in 91 and 82 blood samples at baseline and at 7 to 14 days after initiation of therapy, respectively (Table 1 and Supplementary Table S1). Results were not available from 11 samples at baseline and 20 samples at 7 to 14 days due to insufficient blood volume and clotted blood samples (Supplementary Fig. S1). Using the CTC/mL equivalent for the CellSearch standard cutoff, 30 (33%) and 28 (34%) patients had ≥0.67 CTC/mL at baseline and 7 to 14 days, respectively (Table 1).

**Concordance of enumeration methods**

Of the 102 patients, 85 (83%) and 75 (74%) had both CellSearch and IE/FC enumeration data at baseline and at 7 to 14 days, respectively. Significant correlations between the two assays were observed both at baseline (Pearson = 0.91; Spearman = 0.62; P < 0.0001) and at 7 to 14 days (Pearson = 0.88; Spearman = 0.53; P < 0.0001; Figure 1).

Kappa statistics was calculated to assess the concordance of CTC status (i.e., CTC-positive or CTC-negative) between the two assays. Good to moderate agreements were observed at different time points using the predefined cutoffs, ≥0.67 CTC/mL and ≥1 CTC/mL (Table 2).

**Association of CTC results with clinical outcomes**

We examined the association of CTC results with TTP and OS. Supplementary Table S1 shows the median TTP and the 95% CIs for different assays and cutoffs. CTC status by both methods at baseline was not predictive for TTP (data not shown). However, at 7 to 14 days, CTC status by CellSearch using the ≥5 CTC/7.5 mL cutoff was significantly associated with TTP (P = 0.0153), whereas a trend was observed for the ≥1 CTC/mL cutoff (P = 0.0527). Interestingly, CTC status at 7 to 14 days via IE/FC using either cutoff was significantly associated with TTP (P = 0.03; Figure 2). However, in absolute terms, this was a difference between 1.4 and 2 months, which is of uncertain clinical significance in this patient population.

Supplementary Table S1 shows the median OS and the 95% confidence intervals for the different assays and cutoffs. CTC status by CellSearch or by IE/FC (Figure 3) at both time points was significantly associated with OS regardless of cutoff (CellSearch: P ≤ 0.0001 at baseline and P < 0.0001 at 7 to 14 days; IE/FC: P ≤ 0.0476 at baseline and P ≤ 0.0086 at 7 to 14 days). As expected, patients with higher CTC counts at either time point (i.e., CTC-positive) had shorter survival, in general 3 to 6 months.
Table 2. Concordance of CTC status (CTC-positive vs. CTC-negative) between CellSearch and IE/FC at baseline and 7 to 14 days after initiation of therapy using the cutoffs, ≥0.67 CTC/mL and ≥1 CTC/mL.

<table>
<thead>
<tr>
<th>IE/FC</th>
<th>CellSearch</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>≥0.67 CTC/mL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Positive: 25, Negative: 37</td>
<td>Positive: 28, Negative: 57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive: 6, Negative: 21</td>
<td>Positive: 21, Negative: 64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive: 6, Negative: 21</td>
<td>Positive: 64, Negative: 21</td>
</tr>
<tr>
<td>7-14 days</td>
<td>≥0.67 CTC/mL&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Positive: 18, Negative: 24</td>
<td>Positive: 25, Negative: 75</td>
</tr>
<tr>
<td></td>
<td>≥1 CTC/mL&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Positive: 15, Negative: 8</td>
<td>Positive: 21, Negative: 64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive: 6, Negative: 21</td>
<td>Positive: 64, Negative: 21</td>
</tr>
</tbody>
</table>

<sup>a</sup>Kappa = 0.63 (good agreement); SE of kappa = 0.08; 95% CI, 0.47–0.80.
<sup>b</sup>Kappa = 0.57 (moderate agreement); SE of kappa = 0.10; 95% CI, 0.37–0.77.
<sup>c</sup>Kappa = 0.49 (moderate agreement); SE of kappa = 0.11; 95% CI, 0.27–0.70.
<sup>d</sup>Kappa = 0.50 (moderate agreement); SE of kappa = 0.11; 95% CI, 0.28–0.72.

for those with unfavorable CTC counts, and 12 months for those without.

Next, we examined the association between survival and changes in CTC numbers between time points (baseline and at 7 to 14 days). Supplementary Table S2 shows the median TTP and OS and the 95% CIs for different groups based on CTC status, i.e., patients with favorable (<cutoff>) and unfavorable (≥cutoff) CTC status at both time points or those who experienced a conversion in CTCs status between time points (favorable to unfavorable or vice versa). A statistically significant difference in TTP was detected among groups based on the CellSearch method using the ≥5 CTC/7.5 mL cutoff (P = 0.0320) but not for ≥1 CTC/mL cutoff (P = 0.1475). There was no significant association between TTP and changes in CTC numbers via IE/FC using either cutoff (P = 0.08; Figure 4; Supplementary Table S2). There was a statistically significant association between changes in CTC numbers between time points and OS for both assays using the predefined cutoffs (Figure 4; CellSearch: P < 0.0001; IE/FC: P ≤ 0.0006). Patients with unfavorable CTC status at both time points and those who converted from favorable to unfavorable had shorter median OS (Supplementary Table S2).

Multivariate analysis and hazard ratios

We used Cox proportional hazard models to determine the comparative prognostic value of CellSearch and IE/FC dichotomized measurements at baseline or days 7 to 14 on TTP and on OS. The cutoff of ≥1 CTC/mL by CellSearch at 7 to 14 days had borderline significant prognostic value (P = 0.07) relative to TTP and OS. Comparisons revealed that IE/FC measurements had lesser prognostic value relative to TTP or OS (Supplementary Table S3).

We used Cox proportional hazard models to determine the prognostic value of CTC status, i.e., favorable (<cutoff>) and/or unfavorable (≥cutoff) at baseline and 7 to 14 days using the predefined cutoffs from CellSearch and IE/FC. The group with favorable CTC status at both baseline and 7 to 14 days was used as reference to estimate the risk of progression or death in patients belonging to other groups. For TTP, only the cutoff of ≥1 CTC/mL by CellSearch had significant prognostic value in patients who experienced a conversion from favorable to unfavorable CTC.
status [Model #1, \( P = 0.036; \) hazard ratio \( (HR) = 2.6 \)]. For OS, unfavorable CTC values at both time points using either cutoff for CellSearch \( (P < 0.0002; HR > 3.8) \) and IE/FC \( (P < 0.0049; HR = 2.7) \) had significant prognostic value (Supplementary Table S4).

### Discussion

CTC enumeration by methods such as CellSearch has been shown to have prognostic significance in metastatic breast cancer (1–9). Although an array of new technologies has recently been developed for CTC capture or enumeration (19), it remains unclear whether newer tests are in fact measuring the same cells. Also, different methods for detection of disseminated tumor cells in bone marrow have led to conflicting results, representing a major obstacle to greater clinical use (20). A key question remaining is whether different methods correlate when testing the same samples (21).

The CellSearch system (22), an FDA-cleared methodology, has been the most widely used CTC technology in clinical trials (12) as well as clinical practice. CellSearch currently uses the established cutoff of \( \geq 5 \) CTCs per 7.5mL for prognostication in metastatic breast cancer. Our CellSearch CTC enumeration results revealed a prevalence \( (\text{i.e., } 44\% \text{ with } \geq 5 \text{ CTCs per 7.5mL of blood at baseline}) \) that is consistent with other CTC enumeration studies in patients with metastatic breast cancer (2, 3, 23–25).

Direct comparison of IE/FC and CellSearch in enumerating CTCs in patients with TNBC revealed significant concordance between the two assays. Most importantly, CTC numbers by either assay were significantly correlated with OS. Our results confirmed findings from other CellSearch studies showing that baseline and
follow-up CTC numbers in patients with metastatic breast cancer, including TNBC, are correlated with OS (3, 26). In this study, the strength of association between CTC levels and OS was evident from the highly significant $P$ values ($P < 0.001$) despite the limited sample size. Baseline CTCs levels were not associated with TTP, possibly due to the inefficacy of the treatment. However, we found that CTC numbers at 7 to 14 days after initiation of therapy were correlated with TTP, suggesting that CTCs may be an early marker of response to targeted therapy. Our data suggest that CTC counts at 7 to 14 days after initiation of therapy may be a more robust marker of risk of progression than baseline counts. Assessment of CTCs within 1 to 2 weeks after starting targeted therapy may therefore allow even earlier determination of futility or efficacy.

It is notable that in this study two independent assays showed highly concordant results. This is, to our knowledge, the first report of a new CTC assay showing both strong concordance with the well-established CellSearch method along with prognostic significance in a new cohort. These findings were likely due to the shared features of these two techniques, including the use of an immunomagnetic capture based on EPCAM expression for enrichment of putative CTCs. Also, both use negative selection for leukocytes using anti-CD45 and positive selection.

Figure 4.
Survival and CTC status at baseline and 7 to 14 days after initiation of therapy. TTP (left) and OS (right) in patients with triple-negative metastatic breast cancer for those with favorable (cutoff) and unfavorable (≥cutoff) CTC status at both time points or those who experienced a conversion in CTCs status between time points (favorable to unfavorable or vice versa). CTC status were determined via CellSearch or IE/FC using the cutoffs, ≥5 CTC per 7.5 mL (or ≥0.67 CTC/mL) and ≥1 CTC/mL. The probability of survival and survival in months are plotted on the $y$ and $x$ axes, respectively.
nucleated cells using nucleic acid dyes. EPCAM-based CTC detection methods, like CellSearch and IE/FC, may fail to detect tumor cells that have low expression of EPCAM. The clinical significance of EPCAM-low CTCs, including those with mesenchymal phenotype (27), remains unclear.

Although the clinical utility of CTC enumeration in improving patient outcomes has yet to be demonstrated, molecular characterization of CTCs can provide further insights into the biology of these cells and may open new avenues for personalized therapy. Direct molecular characterization of CTCs requires further purification beyond current enrichment strategies, which retain a considerable amount of leukocytes (11). For example, CTC enriched samples obtained via modified versions of the CellSearch method have been subjected to molecular profiling (28–31). In addition, some methods may provide relative advantage in terms of ability to isolate CTCs usable for genomic and expression analysis (11).

This study demonstrates the feasibility of CTC enumeration via IE/FC. Although the IE/FC assay may be considered an alternative approach to CTC enumeration, it is perhaps most useful as a platform for CTC isolation and molecular profiling (13, 17, 18, 32).

We have recently reported that the IE/FC method can be used for complete isolation and DNA/RNA profiling of highly pure CTCs from patients with advanced cancer (13, 17, 18, 32). Molecular profiling via genome-wide copy-number analysis confirmed the malignant nature of the isolated cells. For example, breast CTCs showed copy-number aberrations (e.g., gains in 1q and 8q and losses in 8p and 16q) consistent with genomic alterations that are frequently observed in primary breast cancers (33). Interestingly, CTCs and primary tumor obtained from the same patient clearly showed clonal-relatedness (13, 18). We also have recently demonstrated the feasibility of RNA-based expression profiling of CTCs isolated using IE/FC (32). Transcriptional analysis of CTCs showed upregulation of mRNA coding for bulky glycoproteins consistent with metastatic phenotype (32).

To our knowledge, this is the largest single clinical trial involving the enumeration of CTCs in TNBC. Other groups have also tested the prognostic value of CTC counts in TNBC (3, 26); however, our study is unique because it has the benefit of involving standardized protocol treatment in the context of a therapeutic clinical trial. In addition, we enumerated CTCs at a much earlier time point (7 to 14 days) compared with other studies (21 to 30 days; refs. 1, 3).

Treatment of TNBC is limited to chemotherapy as there are no targeted therapies identified to date for this biologic subtype (14). Metastatic TNBC is associated with short progression-free survival and OS (34); therefore, identifying nonresponders at an early time point during therapy could possibly aid clinicians in moving patients to an alternative treatment regimen. Although one recent clinical trial failed to demonstrate improved clinical outcomes in MBC using early CTC-guided treatment change (10), it remains unanswered whether this approach may show improved outcomes in a more focused study design, such as in TNBC or with specific therapies. Monitoring of CTCs may become a useful tool in identifying and stratifying prognostic subgroups for clinical trials, as well as in guiding treatment decisions to minimize exposure to expensive, and potentially toxic therapy that is unlikely to provide benefit. Further validation of the clinical utility of CTC enumeration in larger clinical trials of TNBC is warranted.

In summary, we demonstrated the concordance between the IE/FC and CellSearch methods as well as the prognostic impact of CTC detection and enumeration for both assays in TNBC. With the emergence of CTCs as a useful biomarker in the clinic, along with novel CTC detection assays in different phases of development, performing comparative analyses is essential in establishing the clinical validity of these assays.

Disclosure of Potential Conflicts of Interest
J.W. Park reports receiving speakers bureau honoraria from and has provided expert testimony regarding breast cancer treatment for Genentech, and has ownership interests (including patents) in and is a consultant/advisory board member for Merrimack Pharma. No potential conflicts of interest were disclosed by the other authors.

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Study supervision: L.A. Carey, J.W. Park, H.S. Rugo

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References
CTC Analysis in Triple-Negative Breast Cancer


Correction: Circulating Tumor Cell Analysis in Metastatic Triple-Negative Breast Cancers

In this article (Clin Cancer Res 2015;21:1098–105), which was published in the March 1, 2015, issue of Clinical Cancer Research (1), Fig. 1A is identical to Fig. 1B. The authors have supplied us with the correct version of Fig. 1A (below). Figure 1B remains unchanged. In addition, a unit in Fig. 2A was incorrectly printed as "<5CTC/7.5 mL." It should read "≥5CTC/7.5 mL." The corrected figure is below. Finally, units in Figs. 2C, 3E, and 3G were incorrectly printed as "0.67CTC/7.5 mL." They should read "0.67CTC/mL." These corrected figures are also below. The authors regret this error.
Reference


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