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FISH-based determination of HER2 status in circulating tumor cells isolated with the microfluidic CEE™ platform

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Determination of HER2 status in breast cancer patients is considered standard practice for therapy selection. However, tumor biopsy in patients with recurrent and/or metastatic disease is not always feasible. Thus, circulating tumor cells (CTCs) are an alternative source of tumor cells for analysis of HER2. An antibody cocktail for recovery of variable, high- and low-, EpCAM-expressing tumor cells was developed based on FACS evaluation and then verified by CTC enumeration (based on CK and CD45 staining) with comparison to EpCAM-only and with CellSearch® ($n = 19$). *HER2* fluorescence in situ hybridization (FISH) on all (CK+ and CK−) captured cells was compared to HER2 status on the primary tumors ($n = 54$) of patients with late stage metastatic/recurrent breast cancer. Capture of low EpCAM-expressing tumor cells increased from 27% to 76% when using the cocktail versus EpCAM alone, respectively. Overall, CTC detection with the OncoCEE™ platform was better compared to CellSearch® (68% vs. 89%, respectively), and a 93% concordance in HER2 status was observed. *HER2* FISH analysis of CK+ and CK− CTCs is feasible using the CEE™ platform. Although larger clinical studies are warranted, the results demonstrate adequate sensitivity and specificity as needed for incorporation into laboratory testing.

Keywords Microfluidic, CTC, HER2, FISH

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Breast cancer (BC) is a heterogeneous disease, encompassing a number of distinct biological properties that are associated with specific morphological and immunohistochemical features and clinical behavior. The only means to classify invasive breast carcinomas for many decades has been based on histological type, grade, and expression of hormone receptors (1–4). More recently, following the success of the trastuzumab adjuvant clinical trials, characterization of HER2 expression has become an integral part of the pathological workup for BC patients. Oncologists categorize BC patients into three main groups: (1) those whose tumors exhibit the presence of hormone (estrogen and progesterone) receptors and who are subsequently managed with various estrogen receptor (ER) targeted therapies ± chemotherapy; (2) those with HER2-positive tumors (HER2+), in which amplification of the *HER2* gene is demonstrated, and who will receive the HER2-directed monoclonal antibody trastuzumab or, in some situations, lapatinib (a dual tyrosine kinase inhibitor targeting HER2 and epidermal growth factor receptor [EGFR]); and (3) those with tumors that do not show evidence of either (1) or (2) above, otherwise referred to as triple negative (negative for ER, progesterone receptor [PgR], and HER2) patients, for whom chemotherapy is often the only therapy available (5).

In BC, if the cancer recurs, enlarges, or spreads while the patient is on a hormonal or HER2-directed therapy, that therapy is often stopped and/or altered. It is assumed the tumor may have changed its hormone receptor and/or HER2 status (e.g., HER2-negative [HER2−] tumors can transform/progress to positive status over the course of treatment in response to the selective pressure of the treatment and as a result of clonal expansion), and a different therapeutic

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approach should be considered (6). Selection of subsequent therapies is again dictated by HER2 and ER/PgR status, indicating the need to frequently retest patients/tumors for such changes.

The presence of circulating tumor cells (CTCs) in the peripheral blood of BC patients has long been associated with metastasis and poor survival (7–9). Though CTCs may serve as a surrogate for metastatic tumor tissue and offer an opportunity to perform tumor-based correlative studies without subjecting patients to the risk of serial biopsies (5,7,10–12), studying them has been challenging due to their extreme rarity in comparison with hematologic cells (about 1 tumor cell per 1 billion blood cells). As a result, technical advances now make it possible for the detection of CTCs in whole blood; yet, current techniques are limited in their capture efficiency and the ability to allow detailed phenotypic and genotypic evaluation of the CTCs (11–13). The only commercially available CTC test (CellSearch®; Veridex LLC, North Raritan, NJ) has permitted detection of CTCs in the blood of patients with metastatic disease (7,8,12,14) but with an overall informative rate or frequency (positive detection of CTCs) of $\sim$50%, only in late-stage patients (15). Given that CellSearch® is limited to capture and detection of epithelial-derived CTCs based on EpCAM and cytokeratin, respectively, additional CTCs, though likely present, fail to be captured and/or detected because their expression of these molecules is either too low or absent. Thus, to use CTCs clinically in a more comprehensive and sensitive manner, there remains a need for a methodology that can efficiently enrich, capture, and subsequently improve the detection rates of CTCs. We report the technical validity of a microfluidic-based, laboratory-developed test, OncoCEE™ (cell enrichment and extraction technology; Biocept Inc., San Diego, CA), for capture, enrichment, and subsequent molecular evaluation of *HER2* amplification status in CTCs by fluorescence in situ hybridization (FISH).

Patients and methods

Laboratory information and samples collected

Patients with advanced-stage BC were enrolled from June 2010 to November 2010. Peripheral blood was collected under appropriate third-party institutional review board–approved protocols (ConversantBIO, Huntsville, AL; BioOptions, Fullerton, CA) and delivered to Biocept's CLIA/CAP accredited laboratory. All patients involved in this study provided written informed consent. In comparison to the CellSearch® System, two tubes of blood from each of the 19 patients with BC was collected in vacutainer tubes containing ACD (1.5 mL acid-citrate-dextrose solution) and an anti-clumping reagent (CEE-Sure™, Biocept Inc.). The CellSearch® assay was performed at an independent medical laboratory (Genoptix Medical Laboratory, Carlsbad, CA). Results from the higher total number of CTCs from a single tube of blood on the OncoCEE™ platform was used for the comparison to CellSearch®. For evaluation of HER2 concordance between CTCs and primary tumors, 20 mL ($n = 13$) and 30 mL ($n = 41$) of blood was collected from 54 patients into 10 mL ACD vacutainer tubes (BD, Franklin Lakes, NJ), followed by the immediate injection of the CEE-Sure™ anti-clumping reagent directly into the vacutainer tubes. Medical records were reviewed for determination of HER2 status in the primary tumor upon initial diagnosis. Samples were stored at room temperature and processed within 24 hours of collection. As required for laboratory-developed FISH-based tests, *HER2*-positive cell lines (BT474 and SKBr3) and negative control blood from normal donors ($n = 5$) was initially obtained and used to demonstrate analytical sensitivity/specificity of the HER2/17 probe set on 1,000 tumor and 2,500 normal interphase cells using OncoCEE™ within Biocept's CLIA/CAP accredited laboratory. In addition, both positive and negative controls were used with each batch of patient samples undergoing *HER2* FISH analysis.

Cell separation

Leucosep tubes (Greiner Bio-One, Monroe, NC) utilizing a Percoll density gradient method were used to recover the peripheral blood mononuclear cell (PBMC) fraction. An Fc blocker (100 ug/mL) and a capture antibody cocktail (1 μg/mL of each antibody in the cocktail: EpCAM and Trop-2 [BD Biosciences, San Diego, CA]; c-Met [R&D Systems, Minneapolis, MN] and folate binding protein receptor [Istituto Nazionale dei Tumori, Milan, Italy]; N-Cadherin [Sigma-Aldrich, St. Louis, MO]; CD318, MSC, and HER2 [BioLegend, San Diego, CA]; Muc-1 [Fitzgerald, Acton, MA]; and EGFR [Santa Cruz Biotechnology, Santa Cruz, CA]) were added for 30 minutes at room temperature to the recovered PBMC fraction. Following a wash and centrifugation, a biotinylated secondary antibody was added for 30 minutes at room temperature. The cell suspension was washed three times with PBS/EDTA buffer with centrifugation at 400 *g* for 10 minutes. The final pellets were subsequently applied to the OncoCEE™ microchannels.

OncoCEE™ microchannel technology

OncoCEE™ microchannel technology has been described previously (16). Briefly, OncoCEE™ microchannels were manufactured at Biocept Inc. Each microchannel consists of a roughly rectangular chamber (40 mm × 12 mm × 55 microns) in which approximately 9,000 variable diameter posts are mathematically placed to disrupt laminar flow and maximize the probability of contact between the target cells and the posts, which are derivatized with streptavidin, resulting in their capture. Each microchannel was attached to a precision syringe pump (Biocept Inc.) to pull fluid through the microchannel. The cell fraction was run through the microchannel, and the captured cells were stained with a mixture of anti-cytokeratin 7/17 (clone C-46), 18 (clone DA/7), 19 (clone A53-B/A2), and pan-cytokeratin (clone C-11) (BioLegend, San Diego, CA) antibodies labeled with AlexaFluor-488 (Life Technologies, Grand Island, NY). Cells were simultaneously stained with anti-CD45 labeled with Alexa-594 (Life Technologies, Grand Island, NY). The transparent microchannels underwent immediate manual microscopic analysis for enumeration of CK+/CD45−/DAPI+ (criteria for CTC identification), CK−/CD45+/DAPI+ (criteria for background white blood cells), and CK−/CD45−/DAPI+ (possible CTCs that lack CK) cells, with images and X/Y coordinates recorded using Olympus Bx51 fluorescent microscopes equipped with appropriate filters and the FISH

imaging system v5.2 (Metasystems GmbH, Altlussheim, Germany).

Fluorescence in situ hybridization

Following CTC enumeration, the OncoCEE™ microchannels were processed for multicolor FISH using the FDA-approved PathVysion HER2 DNA Probe Kit (centromere 17−specific probe, CEP 17− Spectrum Green, and locus-specific HER2 probe, Spectrum Orange; Abbott Molecular, Abbott Park, IL). Evaluation of FISH signal patterns was performed on both CK+/CD45− and CK−/CD45− CTC populations. The ratio of HER2:CEP17 was calculated, and a ratio >2.2 in any CK+/CD45− or CK−/CD45− cell was regarded as positive for *HER2* gene amplification.

Cell lines and flow cytometry

MCF10A (ATCC, CRL-10317), MDA-MB-231 (ATCC, HTB-26), ZR75-1 (ATCC, CRL-1500), MCF-7 (ATCC, HTB-22), BT474 (ATCC, HTB-20), MDA-MB-468 (ATCC, HTB-132), SKBr3 (ATCC, HTB-30), and MDA-MB-134 (ATCC, HTB-23) BC cell lines were cultured according to American Type Culture Collection (ATCC) recommendations, verified by morphology, growth curve analysis, and tested for mycoplasma. Measurement of surface antigens targeted by the capture cocktail was performed by incubating trypsin-detached, nonpermeabilized BC cells (listed above) with the indicated mouse anti-human IgG antibodies, followed by incubation with PE-labeled anti-mouse IgG (Sigma-Aldrich, St. Louis, MO), according to a standard flow cytometry protocol. After additional washes to remove excess antibodies, the cells were analyzed on the Accuri C6 flow cytometer (Accuri Cytometers Inc., Ann Arbor, MI). Flow cytometric estimation of the number of antibodies bound per cell was determined by using BD Quantibrite™ PE beads (BD Biosciences), according to the manufacturer's instructions. By using known ratios of PE to antibodies, we converted PE molecules per cell to antibodies per cell.

Statistical analysis

To assess the analytical performance of *HER2* amplification by FISH, the sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the patients involved in this study. Cohen's κ statistic was also calculated to adjust for chance, in order to assess the agreement of HER2 status between the primary tumor and CTCs (17).

Results

CTC capture and detection efficiency using an antibody cocktail

The OncoCEE™ platform technology employs the use of an antibody cocktail for the capture of CTCs in peripheral blood samples. Selection of the 10 antibody cocktail was first based on flow cytometric screening of numerous potential cancer-associated target antigens repeatedly reported in the literature (18). Selection was further based upon minimal cross-reactivity of each antibody to normal healthy blood. Figure 1 illustrates the additive antibody binding levels and corresponding antigen expression levels on several BC cell lines for the chosen antibodies, as compared to EpCAM antigen expressed per cell line.

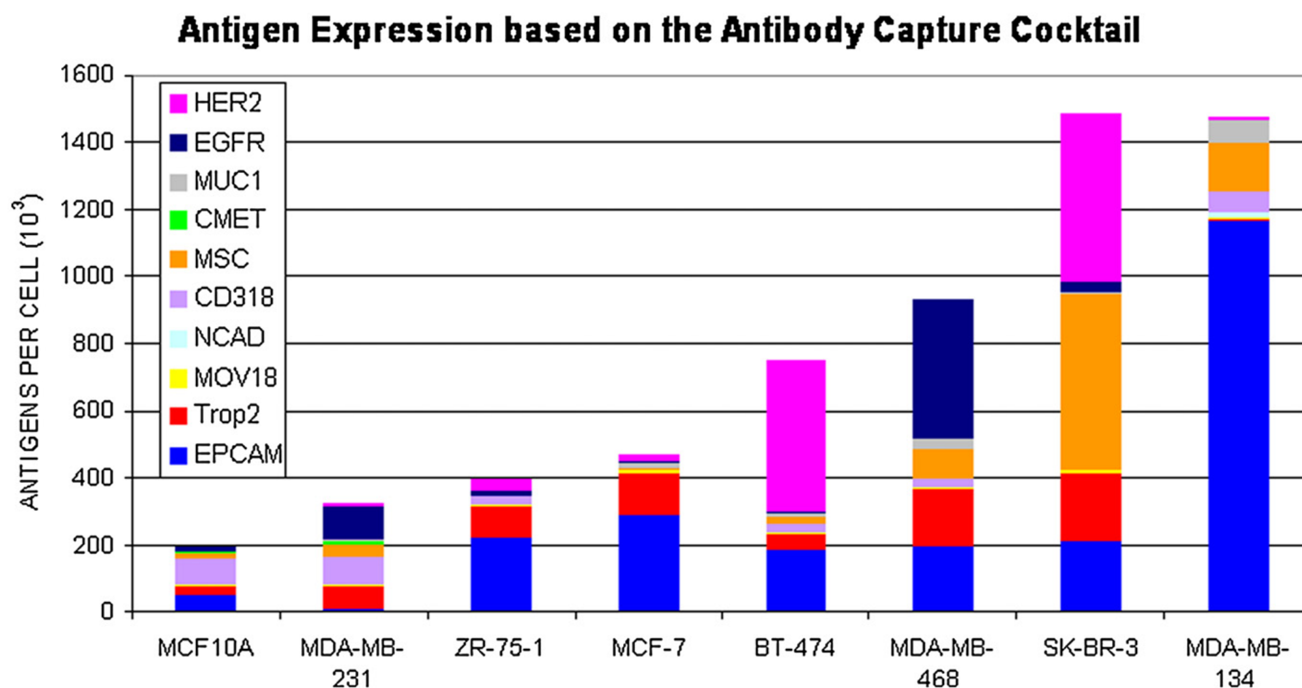

Figure 1 Flow cytometric data. Results illustrating the additive effect of each of the antibodies in the capture cocktail on eight independent BC cell lines.

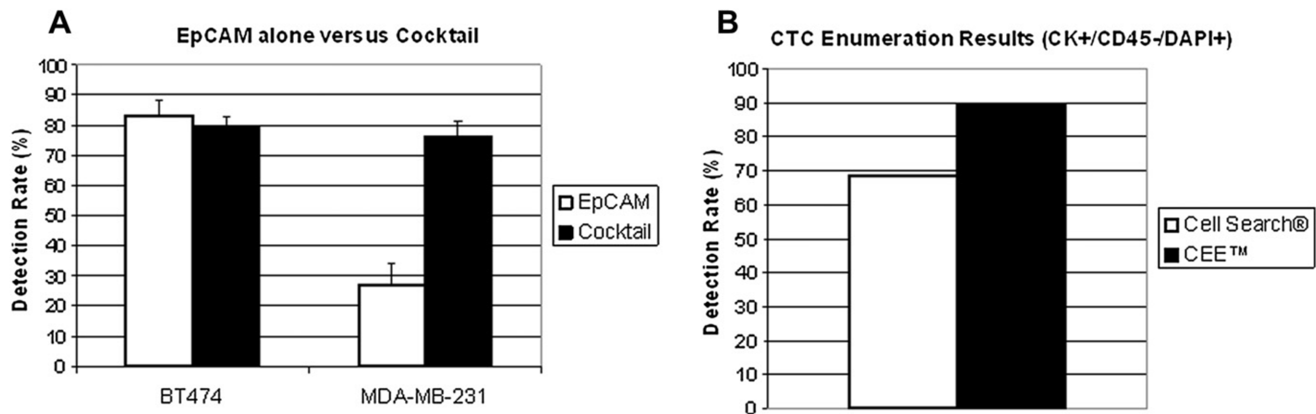

Figure 2 Detection rate of tumor cells based on EpCAM alone or the antibody capture cocktail. (A) Spiked BC cell lines BT474 (high-expressing EpCAM) and MDA-MB-231 (low-expressing EpCAM), showing the benefit of using the antibody cocktail on low EpCAM-expressing cells. (B) Comparison of CellSearch® System, which uses EpCAM for capture of CTCs, to the CEE™ platform, which uses the antibody capture cocktail.

Using this 10-antibody cocktail, we demonstrate more efficient capture of tumor cells expressing low (approximately 12,000 antigens per cell) EpCAM levels as compared to cells expressing high (approximately 184,000 antigens per cell) levels. We compared two cell lines, BT474 (high-expressing EpCAM) and MDA-MB-231 (low-expressing EpCAM), using EpCAM alone as compared to the antibody cocktail with OncoCEE™. Data show that the higher EpCAM-expressing BT474 cells are captured equally using either EpCAM (83% ± 5.2%) alone or the antibody capture cocktail (79% ± 4.1%). However, lower EpCAM-expressing MDA-MB-231 cells are captured well with the cocktail (76% ± 5.0%) but fail to capture efficiently using EpCAM (27% ± 6.9%) alone (Figure 2A).

Table 1 Patient and sample characteristics

Median age, years (range)	64 (29–86)
Ethnicity breakdown, *n* (%)	
White (non-Hispanic)	41 (76)
African American	4 (7)
Hispanic	2 (4)
Asian Pacific Islander	2 (4)
Other	4 (7)
HER2/ER/PgR status, *n* (%)	
HER2−	30 (56)
ER+/PgR+	31 (57)
ER+/PgR−	13 (24)
ER−/PgR−	10 (19)
CTCs detection frequency based on CK+, *n* (%)	
0 CTCs	11 (20)
1–4 CTCs	15 (28)
5–9 CTCs	9 (17)
≥10 CTCs	19 (35)
Cell purity in the OncoCEE™ microchannel, average	
DAPI+	1,924 ± 2,685 cells
DAPI+/CD45−/CK−	~20%
DAPI+/CD45+/CK+	<1%

Abbreviation: n, number.

A pilot study was performed to compare CTC recovery between CellSearch®, which uses only EpCAM for capture, and OncoCEE™, which uses the antibody cocktail. Among 19 BC patients, at least 1 CTC was detected in 13 of 19 (68%) cases on the CellSearch® System and in 17 of 19 (89%) cases using OncoCEE™ (Figure 2B). A median of 3 CK+ CTCs were detected on the CellSearch® System (range, 0–139 CTCs) and 10 CK+ CTCs on the OncoCEE™ platform (range, 0–166 CTCs). The recovery of epithelial CTCs–based cytokeratin detection (CK+/CD45−) was shown to be equivalent (in 13 cases) or better (in 4 cases) with OncoCEE™.

CTC (CK+/CD45−) enumeration

A separate cohort of 54 patients with defined HER2 tissue status were collected and processed for CTC enumeration, followed by *HER2* FISH analysis. Patient characteristics are shown in Table 1. Samples were from late stage IV metastatic or recurrent BC patients sourced through vendors under third-party consent. In this study, there was no significant difference between the number of CK+ CTCs detected in 20 mL of blood ($n = 13$) and 30 mL of blood ($n = 41$) by the OncoCEE™ test using the Mann-Whitney test of medians (7 and 5 CTCs, respectively, $P = 0.87$). Following enumeration, 43 patients were found to have ≥1 CTC identified

Table 2 Concordance of *HER2* FISH status between CTCs isolated using the OncoCEE-BR™ and primary tumor

	CTCs, *n* (%)			
		HER2+[a]		
Primary tumor	HER2−	CK+	CK−	Total, *n*
HER2−	22 (51)	0 (0)	2 (5)	24
HER2+	1 (2)	12 (28)	6 (14)	19
Total	23 (53)	12 (28)	8 (19)	43

Abbreviation: n, number.
[a] HER2+ status is defined as having a ratio of *HER2* FISH signals to centromere 17 ≥ 2.2.

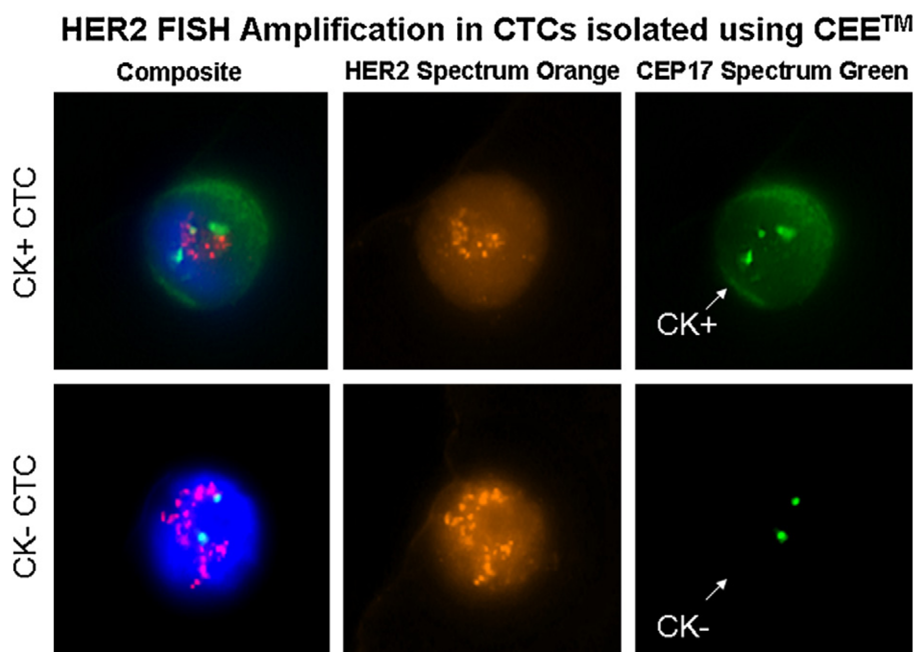

Figure 3 Representative images of *HER2*-amplified cells using the PathVysion probe set. Top panels demonstrate *HER2* amplification (SpectrumOrange) compared to centromere 17 (CEP17; Spectrum Green) in a CK+ cell (arrow indicates Alexa488 green staining). Bottom images demonstrate *HER2* amplification (SpectrumOrange) compared to centromere 17 (CEP17; SpectrumGreen) in a CK− cell (arrow).

based on a staining pattern of CK+/CD45− cells (prevalence of 80% based on CK+ staining). Additional cells were noted to be candidate CTCs based on a CK−/CD45− staining pattern; because these cells failed to stain positively for CD45, they were therefore classified as possible CTCs that may have cytokeratin levels that are too low for visual detection for various reasons, such as down-regulated CK expression through biological processes (i.e., epithelial-to-mesenchymal transition [EMT]). X/Y stage coordinates and images were taken of these cells for subsequent re-location and FISH analysis. A median of 8 CK+ CTCs were detected in the 43 patient samples (range, 1−319 CTCs).

Concordance of HER2 status

Among the 43 CK+ informative cases, an overall concordance of 93% between CTCs and the primary tumor was observed with regard to HER2 status. Concordance was observed in 18 of 19 cases with positive HER2 status (positive predictive value of 90%) and 22 of 24 cases with negative HER2 status (negative predictive value of 96%). There was no difference between the numbers of CK+ CTCs detected in the 19 patients with HER2+ status compared with the 24 HER2− cases (median 5 and 9 CTCs, respectively, $P = 0.37$). Discordance was observed in three cases as shown in Table 2. One patient positive for *HER2* amplification in the primary tumor displayed *HER2*− CTCs in the OncoCEE™ microchannel, and two patients with *HER2*+ CTCs had primary tumors classified as HER2−. An overall test sensitivity of 95% and specificity of 92% was observed. A substantial agreement approaching the range of perfect agreement (0.81−1.00) was found using Cohen's κ statistic (κ = 0.75) for concordance between HER2 status in the primary tumor and CTCs.

An important observation made during the course of this study was that *HER2*-amplified cells enriched using the OncoCEE™ platform were both CK+ and CK− (Figure 3). Similar numbers of *HER2*-amplified cells were detected as CK+ (50%) and CK− (50%) CTCs (mean 4.3 and 4.2, respectively, $P = 0.285$). The presence of CK− CTCs may result from potentially different populations of CTCs captured with the antibody cocktail, or from the presence of CK− epithelial CTCs with low or no expression of CK, or a combination of both. Nevertheless, these results demonstrate reliable FISH detection of a validated tumor biomarker (i.e., *HER2* amplification) in CTCs following enrichment and capture using OncoCEE™, and that this system can be used to confirm a CK−/CD45− cell as a tumor cell.

HER2 FISH sensitivity and specificity was further confirmed with normal peripheral blood lymphocytes and cultured tumor cells with OncoCEE™. *HER2* amplification was not observed among 500 normal blood lymphocytes obtained from each of 5 normal noncancer control individuals ($n = 2500$). Moreover, *HER2*+ cell lines, BT474 ($n = 500$) and SKBr3 ($n = 500$) cells, were captured and subjected to *HER2* FISH analysis for use as positive controls. HER2:CEP17 ratio levels of >10 and 5 were detected in each, BT474 and SKBr3, respectively, and are consistent with published data (19,20).

Discussion

Many studies have reported the identification and characterization of mutations that occur frequently during breast

tumorigenesis, including the overexpression and amplification of human *EGFR2* (*HER2/neu*, *c-erbB2*) (21–25). HER2 status is now widely recognized as an important marker for aggressiveness, and patients that exhibit HER2 overexpression and amplification are treated with the U.S. Food and Drug Administration (FDA)-approved humanized monoclonal antibody trastuzumab (Herceptin; Genentech, San Francisco, CA) (26), which targets this molecule. Although HER2 status determined by FISH and immunohistochemistry (IHC) are correlated, current ASCO-CAP (American Society of Clinical Oncology-College of American Pathologists) guidelines recommend FISH for accuracy, reproducibility, and precision (26). Furthermore, several investigators have reported that HER2 status can vary between the primary tumor and a metastatic site, as well as in CTCs (23,24,27–30). Though monitoring the status of HER2 in CTCs may appear to be a viable option, the technical validity of such a test has not been demonstrated.

The OncoCEE™ platform utilizes an antibody cocktail that may enable capture of a more heterogeneous CTC population (31). The identification of *HER2* gene amplification by FISH in CK+/CD45− as well as CK−/CD45− cells is consistent with the presence of multiple CTC phenotypes. These results strongly indicate that some CK−/CD45− cells are in fact CTCs that have presumably down-regulated CK expression below visible detection levels. Thus, studies that are limited to the use of EpCAM and CK for capture/enrichment and detection of CTCs, respectively, may not observe equivalent concordance of tumor biomarkers such as HER2 between CTCs and the primary tumor. Further, we used more than 7.5 mL of blood for enumeration and *HER2* FISH analysis. It is likely that our observed higher concordance rate as compared to other recent published studies (6,20,29,30,32) results from the combined higher blood volume and the inclusion of CK−/CD45− cells for FISH analysis. A comparison of reported concordance rates is listed in Table 3. Although some discordance between the tumor and CTCs is perhaps expected given tumor heterogeneity, biopsy size, and robustness of the technical assay (especially for IHC), a blood-based CTC assay may offer more reliable testing given the advantages of simple repeat testing and the use of larger blood volumes when needed to ensure informative results. It could be utilized to confirm HER2 tumor tissue testing, especially in tissue-negative cases, to ensure that patients suitable for trastuzumab therapy are not inadvertently missed. Further studies are now under way on the validation of alternative staining methods and detection of CK−/CD45− CTCs. Here, we also show that FISH offers a highly sensitive and accurate approach for identifying cytogenetically abnormal cells likely to be of tumor origin that would subsequently aid in further assay optimization.

Table 3 Comparison of HER2 concordance in CTCs and primary tumor

Overall concordance rate (%)	Number of patients in study	Method of HER testing in CTCs	Reference
93	54	FISH	This study
89	29	ICC and/or FISH	(20)
82	61	ICC	(29)
68	66	ICC and/or FISH	(30)
65	62	ICC	(6)
65	75	FISH	(32)

Clinically, CTCs have been demonstrated to provide measurable prognostic value for advanced BC patients (7,8). In the present study, we report the utility of a microfluidic platform that utilizes the OncoCEE™ technology (Biocept Inc.) for capture, enrichment, and subsequent molecular evaluation of *HER2* gene amplification in CTCs by FISH. Although clinical utility for HER2 staging using CTCs in BC is warranted, the feasibility of a FISH-based *HER2* test on isolated tumor cells from peripheral blood has been demonstrated.

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