Redefining CTCs: Detection of additional circulating tumor cells using an antibody capture cocktail and HER2 FISH.

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ABSTRACT

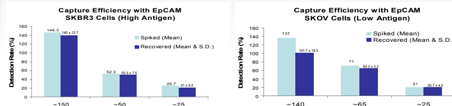
INTRODUCTION: Most circulating tumor cell (CTC) platforms rely on EpCAM for capture and cytokeratin (CK) for detection. However, an important population of cells that display an epithelial-mesenchymal transition (EMT) phenotype will be missed. We report a new strategy to efficiently isolate a more heterogeneous population of CTCs using an antibody cocktail.

METHODS: In the first prospective study, blood (20 mL) was collected from 88 patients diagnosed with various late stage metastatic/recurrent cancer (breast, CRC, lung, prostate) following IRB approval. PBMCs were incubated with a mixture of 10 capture antibodies to target both epithelial and mesenchymal cells. CTCs were subsequently captured in the CEE™ channels and identified with antibodies directed against cytokeratin (CK) and CD45. An additional tube of blood was run on the CellSearch System®. A second prospective IRB approved study involving 54 patients diagnosed with late stage metastatic/recurrent breast cancer was performed using similar detection strategies (CK cocktail mixture and anti-CD45) with the addition of HER2 FISH to determine amplification status among captured CK+/CD45- and CK-/CD45- cells (presumable EMT cells).

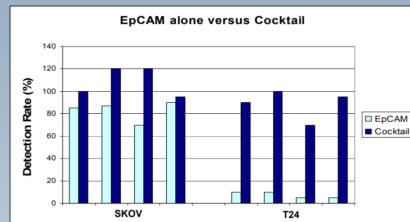
RESULTS: In the first study, overall detection of CK+ cells was greater on the CEE™ platform compared to the CellSearch System® in all solid tumor types tested . In the second study, CK+/CD45- cells were detected in 43 of 54 cases (80%). Among the 43 cases in which CK+/CD45- cells were detected, high concordance (93%) in HER2 status between primary tumor and CTCs was observed with HER2 amplification noted in both CK+/CD45- (50%) and CK-/CD45- (50%) cells.

CONCLUSIONS: We have developed a novel and robust method for CTC enumeration that utilizes a cocktail of antibodies for the detection of a heterogeneous population of CTCs in multiple cancer types. Our findings suggest an important population of CK- cells is being missed by current stain criteria. Data also demonstrate that recovery of CTCs from peripheral blood using the CEE™ platform is efficient and suitable for FISH-based testing.

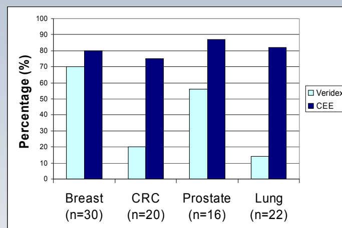
CAPTURE EFFICIENCY



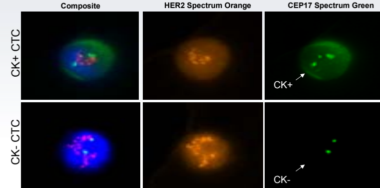
COCKTAIL CAPTURES MORE CTCs



CEE™ v. VERIDEX



HER2 FISH ON CTCs



PATIENT DEMOGRAPHICS

Table 1. Patient Characteristics

Characteristic, n (%)	All Patients n = 54	Patients with ≥ 1 CTC (n = 43)
Median Age, Years (Range)	64 (29-86)	
CTCs based on CK+		
0	11 (20)	
1-4	15 (28)	
5-9	9 (17)	
≥ 10	19 (35)	
HER2+	24 (44)	20 (47)
HER2-	30 (56)	23 (53)
ER+/PgR+	31 (57)	26 (60)
ER+/PgR-	13 (24)	10 (23)
ER-/PgR-	10 (19)	8 (19)
Ethnicity Breakdown		
White (non-Hispanic)	41 (76)	
African American	4 (7)	
Hispanic	2 (4)	
Asian Pacific Islander	2 (4)	
Other	4 (7)	

Abbreviations: CK = cytokeratin; CTC = circulating tumor cell; ER = estrogen receptor; n = node; PgR = progesterone receptor

Patient characteristics are shown in **Table 1**. Following enumeration, 43 patients were found to have ≥1 CTC identified based on a staining pattern of CK+/CD45- cells (prevalence of 80% based on CK+ staining).

RESULTS

Table 2. Concordance of HER2 Status Between CTCs isolated using the OncoCEE-BR™ and Primary Tumor

Primary Tumor	CTCs, n (%)		
	HER2-	HER2+	Total, n
HER2-	22 (51)	2 (5)	24
HER2+	1 (2)	18 (42)	19
Total	23 (53)	20 (47)	43

Abbreviation: CTC = circulating tumor cell, n = node

Among the 43 CK+ informative cases, an overall concordance of 93% between CTCs and the primary tumor was observed with regards 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 to the 24 HER2- cases (median 5 and 9 CTCs, respectively, p = 0.37). Discordance was observed in three cases as shown in Table 2.

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 the Cohen's k statistic (k= 0.75) for concordance between HER2 status in the primary tumor and CTCs.

DISCUSSION

The CEE™ platform utilizes an antibody cocktail to enable capture of a more heterogeneous CTC population based on the identification of HER2 gene amplification by FISH in both CK+/CD45- and CK-/CD45- cells.

These results strongly indicate that 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 significant concordance of tumor biomarkers such as HER2 between CTCs and primary tumor.

Though some discordance between 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 the use of larger blood volumes when needed to ensure informative results.

CONCLUSIONS

The CEE™ technology provides a sensitive platform for enhanced capture, detection and characterization of both CK+ and CK- CTCs.

This platform allows for evaluation of HER2 gene amplification status by FISH in intact CTCs within the microchannels at sensitivity and accuracy levels suitable for standardized CLIA-laboratory testing.

METHODS AND MATERIALS

Laboratory Information and Patients Patients with advanced stage breast cancer were enrolled from June 2010 to November 2010. Peripheral blood was collected under appropriate third party institution review board approved protocols (Conversant Laboratories & BioOptions). Veridex testing was performed by Genoptix Medical Laboratory.

Cell Separation Leucosep tubes (Greiner bio-one, Monroe, NC) utilizing a Percoll density gradient method were used to recover the peripheral blood mononuclear cell fraction (PBMC). Fc blocker (100ug/mL) and a capture antibody cocktail (1µg/mL of each antibody in the cocktail) were added for 30 minutes at room temperature to the recovered PBMC fraction. Following a wash and centrifugation, biotinylated secondary antibody was added for 30 minutes at room temperature. Following washing the final pellets were subsequently applied to the CEE™ microchannels.

CTC Enrichment and Detection CEE™ microchannels are manufactured at Biocept, Inc. (San Diego, CA). The cell fraction is run through the microchannel and the captured cells stained with a mixture of anti-cytokeratin antibodies labeled with AlexaFluor-488. Cells were simultaneously stained with anti-CD45 labeled with AlexaFluor-594. The microchannels undergo microscopic analysis for enumeration of CK+/CD45-/DAPI+ (criteria for CTC identification), CK-/CD45+/DAPI+ (criteria for background WBCs) and CK-/CD45-/DAPI+ (possible CTCs that lack CK) cells.

Fluorescent in situ hybridization Following CTC enumeration, the CEE™ microchannels were processed for multi-color 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) and a centromere specific probe to chromosome 8 (CEP 8, Spectrum Aqua, Abbott Molecular, Abbott Park, IL) for use as an internal control for ploidy status. The ratio of HER2:CEP 17 was calculated and a ratio >2.2 in any CK+ /CD45- or CK-/CD45- cell was regarded as positive for HER2 gene amplification.

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