Detection of circulating tumor cells and HER2 gene amplification status in breast cancer using a novel microfluidic platform (Cell Enrichment and Extraction Technology, CEE™)

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

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Savitri Krishnamurthy[1], Farideh Z. Bischoff[2], Steve Mikolajczyk[2], and Anthony Lucci[3]

University of Texas MD Anderson Cancer Center Department of Pathology [1], Surgical Oncology[3], Biocept, Inc., San Diego CA[2]

Abstract

INTRODUCTION: The rarity of circulating tumor cells (CTCs) in blood poses challenges in their detection.The majority of the techniques utilized for the detection of CTCs are limited in their ability to allow detailed phenotypic and genotypic evaluation of the CTCs. In this pilot study, we report the utility of a novel microfluidic platform utilizing cell enrichment and extraction technology (CEETM, Biocept, Inc., San Diego) for detection of CTCs in breast cancer and subsequent evaluation of HER2 gene amplification status of the CTCs by fluorescent in situ hybridization (FISH)

METHODS: Peripheral blood (10 mL) was collected from patients with operable breast cancer in a prospective institution review board approved protocol. Mononuclear cells were recovered using a Percoll density gradient method, incubated with a mixture of 10 primary capture antibodies (cAb) and then introduced into microchannels (CEE channels). The cells in the channels were stained using fluorescent pancytokeratin (CK) in addition to a fluorescent probe to detect cAbs on the cells. Cells were stained for CD45 as a negative control. The enumeration and localization of cAb-CK +, CD45- as well as cAb-CK-, CD45- cells was performed by microscopic examination of the microchannels. Finally the microchannels were processed for multicolor FISH using three direct labeled probes specific to centromere 8 (spectrum aqua), 17 (spectrum green) and HER2 (spectrum orange). The ratio of HER2:CEP17 >2.2 in any CD45 negative cell was regarded as positive for HER2 gene amplification.

RESULTS: Peripheral blood from 25 patients with T1N0 (12), T1N1 (3), T2N0 (3), T2N1 (2), T2N3 (1), T3N0 (1), T4N0 (2), T4N3 (1) showed cAb-CK+, CD45- CTCs in 75% (9/12), 66.6% (2/3), 33.3% (1/3) of the first three, respectively, and in all the patients in the remaining groups. Amongst the different genomic types of the primary tumor, cAb-CK+, CD45- CTCs were detected in 79% (15/19) Luminal A, 66% of Luminal B (2/3), 50% (1/2) of HER+ and 100% (1/1) of triple negative tumors. Overall 5/25 of the primary tumors were HER2 amplified. While cAb-CK+ cells were isolated in 3/5 of these patients, HER2 amplification was detected in a cAb-CK-, CD45- CTC in only one patient with a Luminal B tumor. In addition, HER2 amplified cAb-CK- CTCs were also detected in a single patient with primary tumor negative for HER2. The two patients with HER2+ CTCs were therefore cAb-CK-, CD45-.

CONCLUSION:

1. The cell enrichment and extraction microfluidic technology (CEETM) provides a sensitive platform for enhanced detection and characterization of antibody-CK positive and negative CTCs.
2. This platform allows evaluation of HER2 gene amplification status by FISH in intact CTCs within the microchannels.
3. The utility of this platform for phenotypic and genotypic characterization of CTCs in breast cancer needs to be tested in larger clinical trials.

Introduction

The presence of tumor cells in peripheral blood in patients with solid tumors constitutes a central event in the complicated process of metastasis. Isolation and characterization of circulating tumor cells (CTCs) can be useful for early detection, monitoring therapy, ascertaining prognosis and in advancing our understanding of the biology of metastatic disease. The rarity of CTCs in comparison to other cellular elements of peripheral blood comprising only $1x10^{-6}$ leukocytes poses significant challenges to their detection. A variety of techniques are currently available for detection of CTCs. The different techniques utilize either independent enrichment followed by detection of CTCs, combined enrichment and detection of CTCs or direct detection without any prior enrichment steps. The majority of the techniques that are utilized for the detection of CTCs are however limited in their ability to allow detailed phenotypic and genotypic characterization of the CTCs. Microfluidic platform (CTC- chip) has been reported that allows efficient and selective isolation of CTCs without prior processing or enrichment. In this pilot study, we report the utility of a novel microfluidic platform utilizing cell enrichment and extraction technology (CEE™, Biocept, Inc., San Diego) for detection of CTCs in breast cancer and subsequent evaluation of HER2 gene amplification status of the CTCs by fluorescent in situ hybridization (FISH).

Methods

Peripheral blood was collected from patients with operable breast cancer in a prospective institution review board approved protocol into 10 mL vacutainer tubes containing 1.5 mL acid-citrate -dextrose solution (ACD solution A vacutainers; BD, Franklin Lakes, NJ). Anti-clumping reagent (Cell - SureTM. Biosept) was injected into the vacutainer tubes within 60 minutes, stored at room temperature and processed within 24 hours of collection. Mononuclear cells were recovered from the peripheral blood samples using a Percoll density gradient method in Leucosep tubes. The recovered cells are incubated with Fc blocker (100 µg / mL) and a capture antibody cocktail adjusted to a concentration of 1µg / mL for 30 minutes at room temperature. After centrifugation, secondary antibody was added to the cell pellet, incubated for 30 minutes at room temperature and centrifuged three times at 400 G for 10 minutes following washings with PBS/ Casein/ Arginine/ EDTA. The resulting 1mL cell pellet was then introduced into the cell enrichment and extraction (CEE) microchannels. Each CEE microchannel is attached to a syringe pump. The resuspended cells (300µL) are introduced to the CEE microchannels at a volumetric flow rate of 18 µL/ min. The cells in the CEE microchannels were then subjected to immunofluoresecent staining using AlexaFluor – 488 tagged mixture of cytokeratin antibodies directed against CK 7,17,18,19, AlexaFluor - 594 tagged CD45 antibody. The microchannels were examined under the microscope and cells with the phenotype CK +/ CD45 -/ DAPI + OR CK - / CD 45 - / DAPI + were localized, enumerated and their precise location were recorded so as to allow re-localization of the same cells following FISH analysis. Following enumeration of the CTCs, the CEE microchannels were processed for multi-color FISH using three direct labeled probes (Abbot Molecular) specific to the centromeres of chromosomes 8 (CEP 8- Spectrum Aqua) and 17 (CEP 17- Spectrum Green) and the locus specific HER2 probe (Spectrum orange). The scoring of the signals was performed on both CK+ and CK- CTCs that were enumerated and localized in the microchannels prior to FISH testing. The ratio of HER2: CEP 17 >2.2 in any CD 45 negative CK+ or CK- cell was regarded as positive for HER2 gene amplification.

Results

Peripheral blood was obtained from 25 patients with invasive breast cancer including 12 T1NO, 3 T1N1, 3 T2NO, 2 T2N1, 1 T2N3, 1 T3NO, 2 T4NO and 1 T4N3 patients. We found cAb-CK+, CD45 - CTCs in 9/12 (75%) of T1NO, 2/3 (67%) of T1N1, 1/3 (33%) of T2NO and in all the patients in the remaining groups. Among the different genomic groups of the primary tumor, cAb-CK +, CD45 - CTCs were detected in 15/19 (79%) of Luminal A tumors , in 2/3 (66%) of Luminal B, 1/2 (50%) of HER2 + and in the only patient with triple negative tumor. Five of the 25 patients included in the study were positive for HER2 gene amplification documented by (FISH). We detected cAb-CK+, CD45 - cells in 3/5 of these patients. HER2 gene amplification by FISH was detected in the CTCs in only 1/5 patients with HER2 gene amplification of the primary tumor in a cAb-CK-, CD45 - CTC. In addition, we also encountered HER2 gene amplification in cAb-CK -, CD45 - CTC in a single patient with a Luminal A T3NO primary breast tumor proven to be negative for HER2 gene amplification.

Results

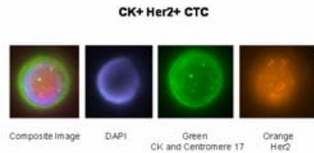

Fig 1:- Illustration of a CK+ and CD45 – CTC, by fluorescence in situ hybridization (FISH). Note the numerous orange signals in the CTC indicating HER2 gene amplification.

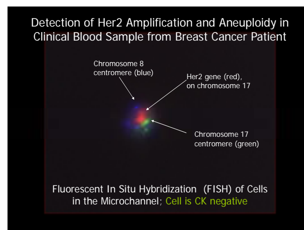

Fig 2:- Illustration of a CK -, CD45- CTC demonstrating HER2 gene amplification by fluorescence in situ hybridization FISH.

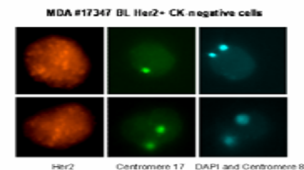

Fig 3:- Illustration of a CK -, CD45- CTC showing HER2 gene amplification by fluorescence in situ hybridization FISH.

TABLE 1: DETECTION OF CIRCULATING TUMOR CELLS (CTCs) IN INVASIVE BREAST CARCINOMA USING CELL ENRICHMENT AND EXTRACTION TECHNOLOGY (CEE™, BIOCEPT)

INVASIVE BREAST TUMOR		CIRCULATING TUMOR CELLS	
TUMOR STAGE	NO. of CASES	NO. of CASES	%
T1 NO	12	9	75
T1 N1	3	2	67
T2 NO	3	1	33
T2 N1	2	2	100
T2 N3	1	1	100
T2 NO	1	1	100
T4 NO	2	2	100
T4 N3	1	1	100

TABLE 2: DETECTION OF CIRCULATING TUMOR CELLS (CTCs) BY THE CELL ENRICHMENT AND EXTRACTION TECHNOLOGY (CEE™, BIOCEPT) IN THE DIFFERENT GENOMIC TYPES OF INVASIVE BREAST CANCER.

INVASIVE BREAST TUMOR		CIRCULATING TUMOR CELLS	
GENOMIC TYPE	NO. of CASES	NO. of CASES	%
LUMINAL A	19	15	79
LUMINAL B	3	2	66
HER2 +	2	1	50
TRIPLE NEGATIVE	1	1	100

TABLE 3: COMPARISON OF HER2 GENE AMPLIFICATION STATUS BETWEEN THE PRIMARY INVASIVE BREAST TUMOR AND CIRCULATING TUMOR CELLS (CTCs). USING THE CELL ENRICHMENT AND EXTRACTION TECHNOLOGY (CEE™, BIOCEPT).

PRIMARY BREAST TUMOR HER2 STATUS	CTCs	HER2 STATUS OF CTCs	
		CK +	CK -
POSITIVE	+	O	+
POSITIVE	O	O	O
POSITIVE	+	O	O
POSITIVE	O	O	O
POSITIVE	+	O	O
NEGATIVE	+	O	+

Conclusions

1. The cell enrichment and extraction microfluidic technology (CEE™) provides a sensitive platform for enhanced detection and characterization of antibody cytokeratin positive and negative CTCs.
2. This platform allows evaluation of HER2 gene amplification status by fluorescence in situ hybridization (FISH) in intact CTCs within the microchannels.
3. The utility of this platform for phenotypic and genotypic characterization of CTCs in breast cancer needs to be tested in larger clinical trials.

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