Detection of HER2 gene amplification in circulating tumor cells and disseminated tumor cells by fluorescence in situ hybridization using OncoCEE™

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Abstract

BACKGROUND: The status of HER2 gene amplification in circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) might provide useful information for monitoring response to trastuzumab therapy, and may provide a basis for consideration of trastuzumab in patients with HER2 negative primary tumors who have HER2 positive CTCs and/or DTCs. The majority of techniques utilized for detection of minimal residual disease are limited in their ability to allow detailed phenotypic and genotypic evaluation of the cells. We report the utility of a microfluidic platform (OncoCEE™ , Biocept, San Diego) for detecting HER2 gene amplification in CTCs and DTCs in patients with non-metastatic breast cancer.

METHODS: Peripheral blood (10mL) and bone marrow (BM) (1-2mL) were collected from patients with clinical stage I-III breast cancer in acid citrate dextrose solution (BD,Franklin Lakes, NJ) and anti-clumping reagent (OncoCEE-Sure™). Mononuclear cells were recovered using a Percoll density gradient method, incubated with a mixture of 10 primary capture antibodies (Abs), introduced into CEE™ microchannels, stained with fluorescent anti-cytokeratin (CK) and anti-CD45 antibodies and finally processed for fluorescence *in situ* hybridization (FISH) using probes specific to centromere 17 (SpectrumGreen) and HER2 (SpectrumOrange). The ratio of HER2:CEP17 were recorded in each case.

RESULTS: Peripheral blood and/or BM from 107 patients (88 BM; 99 blood; 79 matched blood and BM) with T1N0 (51), T1N1 (9), T2N0 (18), T2N1 (6), T2N2 (1), T2N3 (3), T3N0 (5), T3N1 (2), T3N2 (1), T4N0(6), T4N1 (3), T4N3 (2) with HER2+ (n=15) and HER2- (n=92) primary invasive breast tumors were studied. The 15 patients with HER2+ primary tumors had HER2+ CTCs and/or DTCs in 4/15 (27%) cases. HER2+ CTCs and/or DTCs occurred in 23 of 92 (25%) patients with HER2- primary breast tumors.

CONCLUSION:

1. OncoCEE™ provides a sensitive platform for evaluation of HER2 gene amplification of CTCs and DTCs.
2. HER2+ CTCs and/or DTCs occurred in 25% of operable patients with HER2- primary tumors.
3. Discordant HER2 status was contributed mainly by HER2+ DTCs occurring in 18 patients with HER2- primary tumors when a HER2 ratio cut-off of ≥ 2.0 is applied. A HER2 ratio cut-off of ≥2.2 similarly results in discordance involving 15 HER2- primary tumor patients.
4. The clinical significance of evaluating the status of HER2 gene amplification in CTCs and DTCs in the management of patients with breast cancer needs to be evaluated prospectively in larger clinical trials.

Introduction

The occurrence of circulating tumor cells (CTCs) in blood and disseminated tumor cells (DTCs) in bone marrow (BM) in patients with early and advanced breast cancer patients is well recognized. These cells most likely play an important role in the complicated process of metastasis. The detection of these cells is challenging due to their extreme rarity in comparison to other hematopoietic cells. The majority of techniques utilized for the detection of CTCs and DTCs do not allow their detection with a high level of sensitivity. Also, the flexibility in staining and post-enumeration molecular analysis are limited with these techniques. The status of HER2 gene amplification in CTCs and DTCs might provide useful information for monitoring response to trastuzumab therapy, and may provide a basis for consideration of trastuzumab in patients with HER2 negative primary tumors who have HER2 positive CTCs and/or DTCs. The OncoCEE™ microfluidic based platform has been developed and shown to efficiently capture and detect CTCs based on standard cytokeratin(CK)+/CD45-/DAPI+ stain criteria. The system enables sequential recovery of both CK+ and CK- CTCs as demonstrated by fluorescence in situ hybridization (FISH) analysis of the recovered cells. We describe here the utility of the OncoCEE™ device for detecting HER2 gene amplification by FISH in intact CTCs and DTCs in patients with operable breast cancer.

Materials & Methods

Sample Collection: Specimens of peripheral blood (10mL) and BM (1-2mL) were collected from patients with operable breast cancer in a prospective institution review board-approved protocol into 10mL vacutainer tubes containing 1.5 mL acid-citrate-dextrose solution (ACD solution A vacutainers; BD, Franklin Lakes, NJ) and anti-clumping reagent (OncoCEE-Sure™).
Cell Separation: Mononuclear cells were recovered from the peripheral blood and BM samples using a Percoll density gradient method in Leucosep tubes (Greiner bio-one, Monroe, NC). The recovered cells were 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 400G for 10 minutes following washings with PBS/Casein/EDTA. The resulting 1mL cell pellet was then introduced into the cell enrichment and extraction (CEE™) microchannel.
CTC Capture and Staining: Each CEE™ microchannel was attached to a syringe pump. The resuspended cells (800µL) were passed through the microchannels at a volumetric flow rate of 18 µL/min. The cells in the microchannels were then subjected to immunofluorescent staining using an AlexaFluor-488-tagged mixture of cytokeratin antibodies and AlexaFluor-594-tagged CD45 antibody. The microchannels were examined under the microscope and cells with the phenotype CK+/CD45-/DAPI+ or CK-/CD45-/DAPI+ were identified, enumerated and their precise location recorded so as to allow re-localization of the same cells following FISH analysis.
CTC Fluorescent In-situ Hybridization: Following enumeration the CEE™ microchannels were subsequently processed for multi-color FISH using the FDA-approved PathVysion HER2 DNA Probe Kit consisting of two direct-labeled probes (Abbott Molecular, Abbott Park, IL) specific to the centromere of 17 (CEP 17-SpectrumGreen) and the locus specific HER2 probe (SpectrumOrange). The FISH scoring strategy involved relocation of the CK+/CD45-/DAPI+ enumerated CTCs followed by the scoring of all the remaining cells with the phenotype CK-/CD45-/DAPI+. This strategy permitted the scoring of both CK+/CD45- as well as those CK-/CD45- CTCs for the CEP17 and HER2 probes. Visualization and images were taken using the Olympus Bx51 fluorescent microscope (Olympus America Inc, Center Valley, PA) at 400X and 600X magnification. The ratio of HER2:CEP17 in any CK+ or CK- CTC or DTC was recorded.
Tissue Fluorescent In-situ Hybridization: Sections of the primary tumor were fixed in buffered formalin, routinely processed, embedded in paraffin wax and cut at 5µm thickness. Unstained sections were used for performing interphase FISH for HER2 gene amplification using the PathVysion DNA probe kit (Abbott Lab). A ratio of HER2:CEP17 of ≥2.2 (counting of at least 60 tumor nuclei) was regarded as positive for HER2 gene amplification.

Results

MICROFLUIDIC CHANNELS

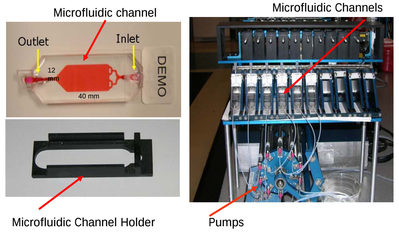


Fig 1: Illustration of the OncoCEE™ microfluidic platform (Biocept, Inc. San Diego) used in the study.

Results

TABLE 1: Details for the clinicopathological characteristics of the patients

TUMOR SIZE	n=107	%
T1	60	56
T2	28	26
T3	8	8
T4	11	10
NODAL STAGE		
NO	80	75
N1	20	18
N2	2	2
N3	5	5
	POSITIVE	NEGATIVE
ESTROGEN RECEPTOR	95/107	12/107
PROGESTERONE RECEPTOR	77/107	30/107
HER2	15/107	92/107

TABLE 2: Detection of HER2 positive CTCs and/or DTCs in patients classified as HER2 negative based on primary tumor. Inclusion based on a HER2/CEP 17 ratio of ≥ 2.0

TUMOR STAGE	PRIMARY TUMOR MARKER STATUS	HER2+ CTCs and/or DTCs (HER2/CEP17 Ratio)
T1N0	ER+,PR-,HER2-	+ (2.5)
T1N0	ER+,PR-,HER2-	+ (3.75)
T1N0	ER+,PR+,HER2-	+ (6.67)
T1N0	ER+,PR-,HER2-	+ (4.0)
T1N0	ER+,PR+,HER2-	+ (8.0)
T1N0	ER+,PR+,HER2-	+ (2.5)
T1N0	ER-,PR-,HER2-	+ (5.0)
T1N0	ER+,PR+,HER2-	+ (5.0)
T1N0	ER+,PR+,HER2-	+ (2.88)
T1N0	ER+,PR+,HER2-	+ (2.0)
T1N0	ER+,PR-,HER2-	+ (2.0)
T1N0	ER+,PR+,HER2-	+ (5.0)
T1N1	ER+,PR+,HER2-	+ (2.0)
T1N1	ER+,PR+,HER2-	+ (2.5)
T1N1	ER+,PR-,HER2-	+ (15.0)
T1N1	ER+,PR+,HER2-	+ (2.29)
T2N0	ER+,PR+,HER2-	+ (12.0)
T2N0	ER+,PR-,HER2-	+ (5.0)
T2N0	ER-,PR-,HER2-	+ (2.0)
T2N0	ER+,PR+,HER2-	+ (2.0)
T2N3	ER+,PR-,HER2-	+ (2.17)
T3N0	ER+,PR+,HER2-	+ (6.0-7.5)
T3N0	ER+,PR+,HER2-	+ (2.0)

Fig 2. HER2 GENE AMPLIFIED CYTOKERATIN POSITIVE CTC

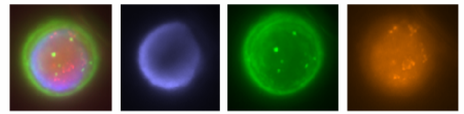
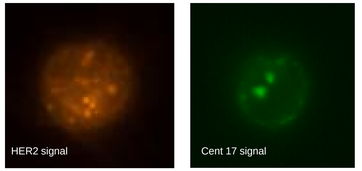


Fig 3. HER2 GENE AMPLIFIED DTC IN BONE MARROW



Conclusions

1. Using a HER2/CEP17 cut-off of ≥ 2.0, HER2 gene amplified CTCs and/or DTCs were detected in 4/15 (27%) patients classified as HER2+ in primary tumor.
2. Among HER2- primary tumor patients (n=92), HER2 amplified CTCs were detected in 6 (6.5%) and DTCs in 18 (20%) cases.
3. An overall HER2 discordance rate of 25% (27 of 107 cases) was observed (based on HER2:CEP17 ratio of ≥ 2.0). The discordance rate is lowered to 18% when a HER2:CEP17 ratio of ≥ 2.2 is applied.
4. Discordant HER2 status was contributed mainly by HER2+DTCs occurring in patients with HER2- primary breast tumors.
5. The clinical significance of evaluating the status of HER2 gene amplification in CTCs and DTCs in the management of patients with breast cancer needs to be evaluated prospectively in larger clinical trials to assess efficacy in treating patients classified as HER2+ by CTC/DTC analysis

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