Detection of HER2-positive Circulating Tumor Cells Using the LiquidBiopsy System in Breast Cancer

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Abstract

This study aimed to use the LiquidBiopsy system and immunofluorescence to measure human epidermal growth factor receptor 2 in circulating breast cancer cells. Seventy-one patients with breast cancer and 107 control provided blood samples. The results indicated that the LiquidBiopsy system may be useful for detecting human epidermal growth factor receptor 2 expression levels on CTC as it was positively correlated with pathologic examination.

Background: Most previous studies of circulating tumor cells (CTCs) are based on the CellSearch platform, but CellSearch has a number of limitations. This study aimed to use the LiquidBiopsy system and immunofluorescence to test the human epidermal growth factor receptor 2 (HER2) status of CTCs in patients with breast cancer. **Materials and Methods:** The LiquidBiopsy system was used to detect HER2-positive ($HER2^+$) cells in whole blood by microfluidic immunomagnetic bead screening and immunofluorescence assay, according to the manufacturer;s instructions. HER2 expression on CTCs was assessed using the Ariol system, calibrated through spiking experiments of 100 cells (BT474, SKBR3, A431, and MDA-MB-231) and 2.5×10^7 white blood cells/mL from healthy donors. Seventy-one patients with breast cancer and 107 non-cancer donors consented to provide blood. **Results:** Based on breast cancer cell lines experiments, $HER2^+$ CTCs were defined as CTCs with HER2 immunofluorescence intensity $\geq$ 3.5 times higher than the CD45 immunofluorescence intensity (100% sensitivity and 99.9% specificity). Among the 71 patients with breast cancer, 31 (43.7%) had $HER2^+$ tumor. Among the $HER2^+$ patients, 41.9% (13/31) were found to be $HER2^+$ based on CTC $\geq$ 1, and 25.8% (8/31) were positive based on CTC $\geq$ 3. In HER2-negative patients by pathologic examination, 1 (2.5%) patient was found to have $\geq$ 3 $HER2^+$ CTCs, whereas 15 (37.5%) patients had $\geq$ 1 $HER2^+$ CTC. $HER2^+$ CTCs were detected at all stages, even in early breast cancer, but the detection rate was higher in metastatic breast cancer. **Conclusion:** This proof-of-concept study strongly suggests that $HER2^+$ CTCs can be detected using the LiquidBiopsy system.

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Keywords: Breast cancer, CTC, HER2, Immunofluorescence, Liquid biopsy

Introduction

Circulating tumor cells (CTCs) are cancer cells that have invaded blood vessels or lymphatics and are carried around the body by the blood.[1] CTCs constitute potential seeds for metastasis because they can reach and implant into distant organs.[2] The clinical importance of CTCs was highlighted in the 1990s with the demonstration of the presence of CTCs early in the disease course.[3] Subsequent research demonstrated the pivotal role of CTCs in the metastatic

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spread of carcinoma.[4] The latest American Joint Committee on Cancer Cancer Staging Manual confirmed the significance of CTC detection and set cM0 (i+) as a stage between M0 and M1.[5] The detection of CTCs using the CellSearch system (Veridex, Raritan, NJ) can predict chemotherapy efficacy.[6-8]

Biopsy of the primary tumor is invasive, cannot be used repeatedly, may miss tumor areas with more aggressive features, and is ineffective for understanding the risk of metastasis, monitoring disease progression, and evaluating treatment effectiveness.[9] Because blood can be easily sampled and because CTCs represent disease activity, "liquid biopsies" can reveal the status of the disease and provide important data for patient management and prognosis.[10]

The clinical use of CTCs is not limited to simply counting the cells. Indeed, the use of molecular tests on CTCs is now possible, and this represents the most important and practical application for individualized medicine. Taking breast cancer as an example, patients with human epidermal growth factor receptor 2-positive (HER2$^+$) CTCs could, possibly, benefit from trastuzumab-based therapies, despite a biopsy suggesting a HER2-negative (HER2$^-$) primary tumor, because a biopsy and sections from a surgical specimen can miss aggressive HER2$^+$ foci arising from specific HER2$^+$ clones.[11,12] Nevertheless, there are still some limitations in CTC-HER2 detection. First, the different methods of detection (immunofluorescence, immunohistochemistry, in situ hybridization) have different sensitivity values.[13,14] Second, there is no consensus about the cutoff value for diagnosis and prognosis.[15-17] Finally, most studies are based on the CellSearch platform because it is the only United States Food and Drug Administration-approved platform currently, but the CellSearch platform has been shown to have a number of limitations. Indeed, the CellSearch platform is based on the restricted phenotypic definition of a CTCs (cells of epithelial origin expressing cytokeratin but not CD45) and so excludes many classes of informative cells because the system only allows for the detection of CK$^+$ CD45$^-$ cells and does not allow customization for the detection of other cellular characteristics, such as the expression of HER2, estrogen receptor, and progesterone receptor.[18-20]

The present study is based on the LiquidBiopsy system (Cynvenio LLC, Westlake Village, CA), which is a platform that purifies CTCs from whole blood with sufficient purity for direct molecular analysis. The present study aimed to use the LiquidBiopsy system and immunofluorescence to test the HER2 status in CTCs in patients with breast cancer. The results could provide an innovative way to determine the HER2 status in patients with breast cancer.

Material and Methods

CTC Capture Method Using the LiquidBiopsy System

The LiquidBiopsy system focuses on the capture and molecular analysis of CTCs, including cells undergoing epithelial mesenchymal transition. The HER2$^+$ cells in whole blood were quantitatively detected by microfluidic immunomagnetic bead screening and immunofluorescence assay, according to the manufacturer's instructions. First, biotin-labeled capture antibodies (including HER2-biotin, Epcam-biotin, and other capture antibodies), fluorescence-labeled HER2 antibody, and streptavidin-labeled magnetic beads were added to the sample to obtain complexes made of the magnetic bead, capture antibody, HER2$^+$ cell, and HER2 detection antibody. In addition, fluorescence-labeled CD45 antibody was added to detect leukocytes. Nuclei staining was used to indicate the intact cells.

HER2 Protein Expression in Cell Lines

Cancer cell lines (BT474, SKBR3, A431, MCF7, MDA-MB-231) were bought from Cell Bank (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China). White blood cells (WBCs) were collected from healthy donors. Trastuzumab was bought from Hoffman-La Roche Pharmaceuticals (Bale, Switzerland). Goat anti-human (Alexa Fluor 488) was bought from Abcam (Cambridge, MA). The HER2-if488 anti-HER2 antibody labeled with Alexa Fluor 488 was from Caprico Biotechnologies (Norcross, GA). The HER2 status in cell lines and WBCs was evaluated by immunofluorescence. Cell lines and WBCs were diluted in binding buffer (phosphate-buffered saline with 50% fetal bovine serum) to 2.8×10^5 cells/mL. Trastuzumab and anti-HER2 antibody were diluted with binding buffer to 8 μg/mL. Then, 100 μL of cells and 100 μL of antibody were added to a 1.5-mL tube and incubated for 30 minutes at 2-8°C. The cells were incubated with goat anti-human (Alexa Fluor 488) for 20 minutes after washing with phosphate-buffered saline twice. The HER2 status was evaluated by analyzing the intensity and ratio using the Ariol DM6000B system (Leica Microsystems, Wetzlar, Germany).

HER2$^+$ CTC Detection

Peripheral blood samples (4 mL) were collected and stabilized with fixative for 24 to 96 hours, and then lysed using lysis buffer. The biotinylated epithelial mesenchymal transition cocktail and streptavidin beads were used for CTC capture. Labeled CTCs were stained with anti-HER2 monoclonal antibodies (HER2-if488), and WBCs were stained with anti-CD45 monoclonal antibodies (CD45-APC). The identification and counting of CTCs were performed using the Ariol DM6000B system (Leica Microsystems). The HER2$^+$ CTCs were defined as nucleated cells lacking CD45 and expressing HER2.

HER2 expression on CTCs using the Ariol system was calibrated through spiking experiments of 100 cells (BT474, SKBR3, A431, and MDA-MB-231) and 2.5×10^7 WBCs/mL from healthy donors. The cell lines were defined as nucleated cells lacking CD45, but with nuclei larger than that of WBCs. HER2$^+$ CTCs were cells with HER2 staining intensity $>$ 2.5-fold of the background.[21] HER2 expression in CTCs was quantified using the formula: HER2 expression = cell HER2 staining (fluorescein isothiocyanate/cell CD45 staining CY5). The formula was entered into the Ariol system. Three independent readers were invited to verify the reproducibility of this method by analyzing 291 BT474 cells, 511 SKBR3 cells, 464 MCF7 cells, 100 A431 cells, 144 MDA-MB-231 cells, and 785 WBCs. The SKBR3 and BT474 cell lines were defined as positive, whereas the MCF7, A431, MDA-MB-231, and WBCs were defined as negative.

The LiquidBiopsy system for the evaluation of the HER2 status was tested by spiking experiments with different concentrations of SKBR3 cells. The recovery of SKBR3 was calculated. Independent blood central image review was performed by Livzon Gene Diagnostics Ltd (Zhuhai, China).

Table 1 HER2/CD45 Fluorescence Intensity Ratio in 5 Breast Cancer Cell Lines

	Median	Range
SKBR3	9.95	3.56-34.23
BT474	15.88	3.49-25.88
MCF7	1.60	0.54-3.44
A431	1.68	1.19-3.64
MDA-MB-231	1.76	1.31-3.58
WBC	1.34	0.49-3.10

Abbreviations: HER2 = Human epidermal growth factor receptor 2; WBC = white blood cells.

Patients With Breast Cancer and Non-cancer Donors

Seventy-one patients with breast cancer and 107 non-cancer donors consented to provide blood. All donors were from the Zhuhai Maternity and Child Care Hospital. The study was approved by the medical ethics committee for Zhuhai Maternity and Child Care Hospital. The inclusion criteria were: (1) 18 to 75 years of age; and (2) stage III/IV breast cancer based on primary tumor pathology. The exclusion criteria were: (1) another primary cancer; or (2) any serious comorbidity such as hepatitis or AIDS. Some patients had received various treatments prior to blood sampling. The pathologic diagnosis of $HER2^{+}$ breast cancer was made on the primary tumor.

Statistical Analysis

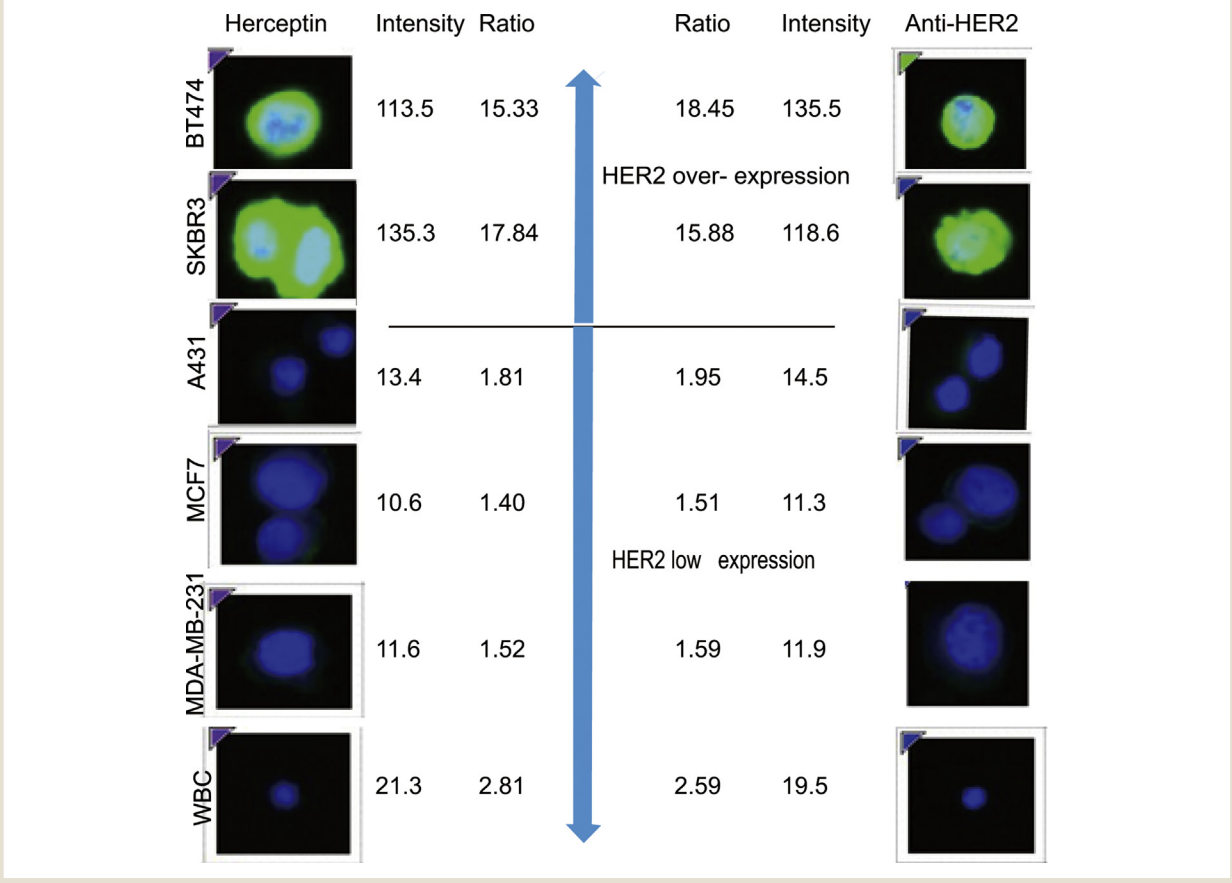
Categorical variables were presented as frequencies and analyzed using the χ^2 test. Continuous variables were presented as medians (range) and analyzed using the Mann-Whitney test. The Pearson correlation test was used to assess the correlations between continuous variables. The receiver operating characteristic curve analysis was used to calculate the cutoff of HER2 staining. All statistical analyses were performed using SPSS 22 (IBM, Armonk, NY) and GraphPad Prism 7 (GraphPad Software Inc, San Diego, CA).

Results

HER2 Staining of CTCs

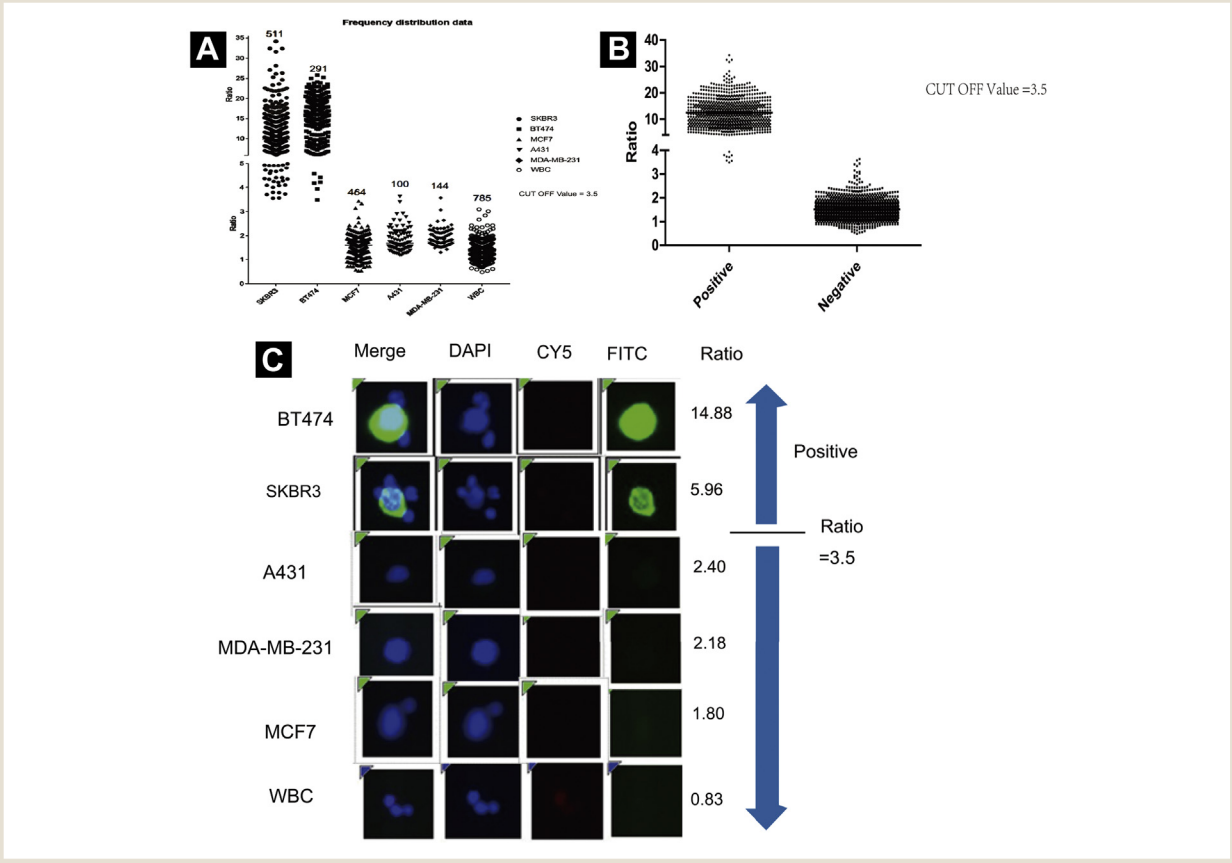
Table 1 and Figure 1 present the HER2/CD45 fluorescence intensity ratio of the 5 breast cancer cell lines. As expected, the SKBR3 and BT474 cell lines ($HER2^{+}$) showed high intensity for HER2/CD45, whereas the $HER2^{-}$ cell lines and WBCs showed a low intensity.

Figure 1 HER2 Fluorescence Intensity in 5 Breast Cancer Cell Lines and WBCs, Using Trastuzumab or an anti-HER2 Antibody. HER2 is Shown in Green and the Nuclei in Blue



Abbreviations: HER2 = Human Epidermal Growth Factor Receptor 2; WBC = white blood cells.

Figure 2 HER2/CD45 Fluorescence Intensity Ratio Based on LiquidBiopsy System in 5 Breast Cancer Cell Lines. HER2/CD45 Cut off Value of 3.5 Adequately Discriminates HER2$^-$ and HER2$^+$ Cells. A, Frequency Distribution by Cell Lines. B, Frequency Distribution by HER2$^-$ and HER2$^+$ Cells. C, Representative Immunofluorescence Images for the 5 Breast Cancer cell Lines



Abbreviations: HER2 = Human epidermal growth factor receptor 2; WBC = white blood cells.

HER2 Expression of 5 Breast Cancer Cell Lines Based on the LiquidBiopsy System

Figure 2 shows the HER2/CD45 fluorescence intensity ratios obtained using the LiquidBiopsy system in the 5 breast cancer cell lines. Using a cutoff value of 3.5 for the HER2/CD45 ratio led to a Youden index of 0.9987, with 100% sensitivity and 99.9% specificity ($P < .0001$).

CTC HER2 Status in Patients With Breast Cancer

Table 2 presents the characteristics of the patients with breast cancer and the univariable analyses with the numbers of CTCs detected using the LiquidBiopsy system. Among the 71 patients with breast cancer, 31 (43.7%) had HER2$^+$ tumor. Among the HER2$^+$ patients, 41.9% (13/31) were found to be HER2$^+$ based on CTC $\geq$ 1, and 25.8% (8/31) were positive based on CTC $\geq$ 3. In HER2$^-$ patients by pathologic examination, 1 (2.5%) patient was found to have $\geq$ 3 HER2$^+$ CTCs, whereas 15 (37.5%) patients had $\geq$ 1 HER2$^+$ CTCs. HER2$^+$ CTCs were detected at all disease stages, but the frequency was higher in metastatic breast cancer. HER2$^+$ CTCs were also found in 2 patients with early breast cancer. Figure 3 presents representative images of the CTCs tested for HER2 using the LiquidBiopsy system. Supplemental Table 1 (in the online version) provides the sensitivity and specificity for different cutoff points. Using a cutoff of $\geq$ 1 cell/4 ml provides higher sensitivity (41.9%), whereas using $\geq$ 3 cells/4 ml provides higher specificity (97.5%).

Discussion

Many previous studies of CTCs are based on the CellSearch platform, but CellSearch has a number of limitations, and it does not allow molecular analysis of CTCs.[18-20] Therefore, the present study aimed to use the LiquidBiopsy system and immunofluorescence to test the HER2 status of CTCs of patients with breast cancer. The results suggest that HER2$^+$ CTCs detected by the LiquidBiopsy system are associated with the HER2 status of breast cancer by pathologic examination. The cell lines experiments showed high sensitivity (100%) and specificity (99.9%) using a HER2/CD45 fluorescence ratio of 3.5.

The present study showed that the LiquidBiopsy system is able to capture breast cancer cells that were spiked among large numbers of WBCs. Based on these experiments, HER2$^+$ CTCs were defined as CTCs with HER2 immunofluorescence intensity that was $\geq$ 3.5

Table 2 Characteristics of the Patients With Breast Cancer and Associations With the Numbers of CTCs Detected Using the Liquidbiopsy System

		Number of $HER2^+$ CTCs, Median (Range)	*P*	$HER2^+$ CTC ≥ 1, n (%)	*P*	$HER2^+$ CTC ≥ 3, n (%)	*P*
Age, y							
< 50	44	0 (0-10)	.084	8 (18.2)	.084	2 (4.6)	.319
≥ 50	27	0 (0-5)		21 (77.8)		8 (29.6)	
Metastasis							
M0	49	0 (0-5)	< .01	9 (18.4)	< .01	1 (2.0)	< .01
M1	22	1 (0-10)		19 (86.4)		8 (36.4)	
Estrogen receptor							
Positive	45	0 (0-10)	.898	19 (42.2)	.898	7 (15.6)	.716[a]
Negative	26	0 (0-4)		11 (42.3)		3 (11.5)	
Progesterone receptor							
Positive	37	0 (0-10)	.465	15 (40.5)	.465	6 (16.2)	.726[a]
Negative	33	0 (0-4)		13 (39.4)		3 (9.1)	
Unknown	1	2	NA	1 (100.0)	NA	0	NA
CTC were measured							
After treatments	59	0 (0-10)	.521	27 (45.8)	.521	9 (15.3)	1.000[a]
Before any treatment	12	0 (0-5)		3 (25.0)		1 (8.3)	
Tissue HER2							
Positive	31	0 (0-10)	.808	13 (41.9)	.808	8 (25.8)	.008[a]
Negative	40	0 (0-4)		15 (37.5)		1 (2.5)	

Abbreviations: CTC = circulating tumor cells; $HER2^+$ = human epidermal growth factor receptor 2-positive.
[a]Fisher exact test.

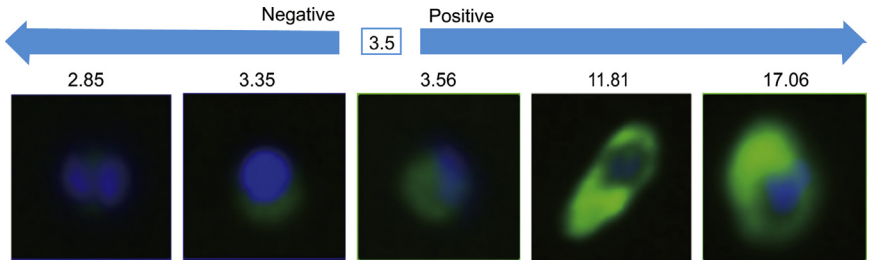
times higher than the CD45 immunofluorescence intensity (100% sensitivity and 99.9% specificity). In addition, the system was able to detect $HER2^+$ CTCs in patients with early breast cancer who had not received targeted therapy. This is supported by previous studies that also showed the presence of CTCs in patients with early breast cancer, albeit using different methods of detection.[22-26]

Nevertheless, the frequency of CTCs was higher in patients with metastatic breast cancer, which is to be expected because cancer cells from the original clone that caused the metastasis probably had features favoring their spread throughout the body.[4] When we take $HER2^+$ CTC counts ≥ 3 as a "strong" HER2 status, significant differences could be found between specimens positive or negative for HER2, providing a possible marker for metastasis.

Of the 71 patients, only 12 had not received any treatment for breast cancer before the study, and the other 59 had been treated with a variety of treatments but no targeted therapy. There was no significant difference in the CTC counts between patients with or without past treatments and this was probably owing to the patients' past treatment not effectively controlling tumor growth. This may also lead to a similar $HER2^+$ CTC count between the 2 groups of patients. Subsequent studies should explore the difference in $HER2^+$ CTC count according to a history of HER2-targeted therapy.

Among the 71 patients with breast cancer, 31 (43.7%) had an $HER2^+$ tumor. Among the $HER2^+$ patients, 41.9% (13/31) were found to be $HER2^+$ based on CTC ≥ 1, and 25.8% (8/31) were

Figure 3 Human Epidermal Growth Factor Receptor 2/CD45 Fluorescence Intensity Ratio of Peripheral Blood Cells in Patients With Breast Cancer



positive based on CTC $\geq$ 3. This is in agreement with previous studies performed using the CellSearch platform, which showed that 30% to 40% of patients with HER2$^+$ tumor at pathologic examination also had HER2$^+$ CTCs.[14,27] Among HER2$^-$ patients by pathologic examination, 1 (2.5%) patient was found to have $\geq$ 3 HER2$^+$ CTCs. This frequency is lower than in a previous study that showed that 20% of patients with HER2$^-$ tumor by pathologic examination had HER2$^+$ CTCs.[28] This discrepancy might be owing to a variety of reasons, including the sample size, the number of sections examined by the pathologists, and cancer treatment history. Moreover, in the present study, 14 (35.0%) HER2$^-$ patients by pathologic examination had 1 or 2 HER2$^+$ CTCs. Although there is not enough evidence to explain the meaning of small numbers of CTCs in cancer treatments, it is worth studying the clinical value of those low counts in a future study. Taken together, these results highlight that pathologic examination might miss some foci of more aggressive disease and that irrespective of the tumor's characteristics, the CTCs are responsible for the metastatic spread, and their characteristics should be taken first into account for patient management.

This also highlights the heterogeneous nature of tumors. Indeed, a tumor may arise from different clones leading to tumor areas with different biological characteristics and aggressiveness. In addition, HER2$^-$ breast cancer cells may spontaneously transform into HER2$^+$ CTCs.[29] The cells responsible for metastatic spread and disease progression should be regarded as the most dangerous ones, and the treatments should be based on those cells. Nevertheless, in the pre-trastuzumab era, it has been shown that HER2$^+$ CTCs in patients with stage I to III breast cancer were associated with a poor prognosis.[26] On the other hand, another study showed that discrepancies in HER2 and estrogen receptor statuses between the primary tumor and CTCs had no impact on the prognosis of metastatic breast cancer.[30] Clinical studies are still necessary to prove this point.

The present study is not without limitations. Indeed, the study population was small and heterogeneous, preventing any immediate clinical application of the results. In addition, no comparison was made with other methods for detecting CTCs, such as the CellSearch system. The present study was a proof-of-concept study for the use of the LiquidBiopsy system for the detection of HER2$^+$ CTCs in patients with breast cancer. At this step, our results cannot be used to determine clinically relevant cutoff points. Additional studies are necessary to determine the prognostic significance of CTCs detected using the LiquidBiopsy system. Nevertheless, the present study indicates that the LiquidBiopsy system can be used to determine molecular characteristics of CTCs. Future studies could examine other characteristics of breast cancer cells such as estrogen receptors, progesterone receptor, and Ki67. This feature of the LiquidBiopsy system could outperform the CellSearch system for individualized medicine because the CellSearch system counts CTCs based on the CK$^+$ CD45$^-$ phenotype.

Conclusion

In conclusion, HER2$^+$ CTCs can be detected using the LiquidBiopsy system. Among the patients with histologically HER2$^+$ breast cancer, 41.9% had $\geq$ 1 HER2$^+$ CTC/4 mL of blood. The cell lines experiments showed high sensitivity (100%) and specificity (99.9%) using a HER2/CD45 fluorescence ratio of 3.5.

Clinical Practice Points

- Many previous studies of CTCs are based on the CellSearch platform, but CellSearch has a number of limitations.
- HER2$^+$ CTCs were detected at all stages, even in early breast cancer, but the frequency was higher in metastatic breast cancer.
- The results indicated that the LiquidBiopsy system may be useful for detecting HER2 expression as the results were associated with the HER2 status by pathologic examination.

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Disclosure

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Supplemental Data

Supplemental table accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2018.10.009.

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Supplemental Data

Supplemental Table 1 Criterion Values and Coordinates of the Receiver Operating Characteristic Curve

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	95% CI	−LR	95% CI
≥ 0	100.00	88.8-100.0	0.00	0.0-8.8	1.00	1.0-1.0		
≥ 1	41.94	24.5-60.9	62.50	45.8-77.3	1.12	0.6-2.0	0.93	0.6-1.4
≥ 2	29.03	14.2-48.0	87.50	73.2-95.8	2.32	0.9-6.2	0.81	0.6-1.0
≥ 3	25.81	11.9-44.6	97.50	86.8-99.9	10.32	1.4-78.2	0.76	0.6-0.9
≥ 4	19.35	7.5-37.5	97.50	86.8-99.9	7.74	1.0-61.0	0.83	0.7-1.0
≥ 5	9.68	2.0-25.8	100.00	91.2-100.0			0.90	0.8-1.0
≥ 11	0.00	0.0-11.2	100.00	91.2-100.0			1.00	1.0-1.0

Abbreviations: CI = confidence interval; CTCs = circulating tumor cells; EMT = epithelial mesenchymal transition; LR = likelihood ratio.