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Detection of Circulating Tumor Cells and Circulating Tumor Stem Cells in Breast Cancer by Using Flow Cytometry

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Abstract

We demonstrated the value of multiparameter flowcytometry in detecting human tumor cells of breast cancer in peripheral blood, which had a sensitivity limit of 10^{-5} and higher specificity compares with real-time polymerase chain reaction (RT-PCR). It was also found that circulating tumor cell (CTC) number was related with TNM stage, metastasis and the overall survival of patients. CTC level was one of the important factors for patients' prognosis. At the same time, we also verified the circulating tumor stem cell (CTSC) was connected with TNM stage by multiparameter cytometry. The detection of CTC and CTSC by multiparameter flowcytometry may be used to diagnose disease at early stage to guide clinical therapy or to predict prognosis. Multiparameter flowcytometry has the potential to be a valuable tool for prognosis assessment among patients with breast cancer in clinical situation in China.

Keywords: circulating tumor cells (CTC), circulating tumor stem cells (CTSC), epithelial-mesenchymal transition (EMT), multiparameter flow cytometry, subtraction enrichment

1. Introduction

MERGEFORMAT breast cancer is the most common cancer in women in developed countries. In developing countries, such as China, the incidence of breast cancer is currently increasing, particularly in larger cities [1]. It is considered to be a systemic disease as tumor cell dissemination at early stage. The major problem of recurrence and death is due to the persistence of minimal residual disease [2]. There is great interest in finding biomarkers in peripheral blood,

which can be sampled at any stage of the disease. Then, the detection of CTCs for monitoring therapy was highly investigated in breast cancer. Circulating cells with the characteristics of tumor cells can be identified in the peripheral blood that is known as circulating tumor cells (CTCs) in many patients with solid tumors of epithelial origin. These cells are present both in patients with metastasis and in those whose tumors are localized [3]. Tumor cells shed into the circulation intermittently which was corresponding with microinvasive events. The first phase of metastatic consists of lessens of tumor cell adhesion, induction of tumor cell motility and local tumor cell invasion [4]. These steps are followed by either spread to circulation in peripheral blood or regional lymph nodes, and locating in secondary organs [5]. Some of these cells generate metastases eventually that can arise many years after therapy of the primary tumor at earlier phase [2]. CTCs also may be related to a half-life probably 1–2.4 h, which means it cannot always exist in circulating [6]. Some authors argued that these cells were predominantly in G0 phase and thus are not replicating [7]; however, they did not exclude the existence that the proliferation of CTCs can occur, although it was a rare event. Considering the half-life of CTCs, the presence of CTCs in the blood could be maintained by a balance between replication and cell death. While apoptosis contributed to a high rate of circulating tumor cells, only a small part of the cells can adhere in second organs through blood vessels that were named as circulating tumor stem cells (CTSCs) [8].

CTCs can be selected with a monoclonal antibody directed against CD45 for negative selection of leukocytes [9–11]. And from this cluster cells, EpCAM (epithelial-cell adhesion molecule) and cytokeratin-8 (CK-8), CK-18, CK-19 (CK-8, CK-18, CK-19 phycoerythrin staining)-positive cells are the target cells, which were known as the marker of epithelial cells. The characterization of CTCs presents a very hot topic in breast cancer research nowadays [12]. It helped to identify diagnosis and provide individual therapies according to the characterization of CTCs [12–14]. The characterization of CTSCs contributed to the identification and targeted therapy in breast cancer in the near future [15]. Molecularly targeted cancer therapies contributed great help which according to the characterization of CTCs especially on patients whose tumors have a particular mutation [16]. Some of the biological properties and the molecular characteristics of CTCs were connected to CTSCs and the genomic profiles have been completed [15]. Following that, the CD44-positive CD24-negative cells with their tumor-initiating ability had been considered as CTSCs [17]. Hence, from CTC, CD44-positive cell and CD24-negative cell are CTSCs. Molecular characterization of CTCs, which is important for the identification of diagnostically and therapeutically relevant for individual therapies, it is difficult to address since they are very rare and the amount of available sample is very limited. Given the properties of the metastatic CTCs, there should be some opportunities for early identification and therapeutic targeting in breast cancer.

Some immunologic procedures, such as immunohistochemistry-based methods and reverse transcriptase polymerase chain reaction (RT-PCR), have been used to detect CTCs in past time [18–22]. However, current methods of detection do not seem to be sensitive or specific enough to apply in clinical [23–25]. Nowadays, we demonstrate the advent of the flow cytometry in the detection of CTCs, which makes a good balance of sensitivity and specificity. In addition,

the procedure of the method was simple and the cost was lower than immunologic technology, which made it possible to apply in clinical.

2. Methods

2.1. Patients

We investigated 45 patients with breast cancer in the Union Hospital in Wuhan during September 2006 and June 2008 with three normal people as negative control. Twenty-five patients had overt metastasis and 20 patients had no sign of overt metastasis. The character-

	All		CTC < 5 (n = 27)		CTC ≥ 5 (n = 18)		P-value ^a
	n	%	n	%	n	%	
Age, years							
Minimum	32	-	32	-	37	-	0.640
Median	50	-	49	-	51	-	
Maximum	74	-	72	-	74	-	
TNM stage							
I	5	13.3	5	100.0	0	0.0	0.033
II	4	8.9	4	100.0	0	0.0	
III	11	24.4	7	63.6	4	36.4	
IV	25	55.6	11	41.7	14	58.3	
Primary tumor sites							
Left breast	24	53.3	14	58.3	10	41.7	0.807
Right breast	21	46.7	13	61.9	8	38.1	
Clinical pathology							
Infiltrating ducta	28	62.2	17	60.7	11	39.3	0.921
Papillary	10	22.2	6	60.0	4	40.0	
Squamous celled	4	8.9	2	50.0	2	50.0	
Mucous cancer	1	2.2	1	100.0	0	0.0	
Medullary	2	4.4	1	50.0	1	50.0	
Diameter of tumor							
≤2.0 cm	13	28.9	9	61.5	4	30.7	0.522
2.0–5.0 cm	27	60.0	16	59.3	11	40.7	
>5.0 cm	5	11.1	2	40.0	3	60.0	
ALND							
Yes	35	77.8	19	54.3	16	45.7	0.143
No	10	22.2	8	80.0	2	20.0	
Metastasis							
Yes	25	55.6	10	40.0	15	60.0	0.002
No	20	44.4	17	85.0	3	15.0	

ALND, axillary lymph node dissection.

Table 1. Patient characteristics.

istics of the patients are shown in **Table 1**, including mean age, TNM phase, histopathology, lymph node status, metastasis and so on. All these patients were incipient and were treated by systemic therapy, including 12 patients cured by cytokine-induced killer cells therapy (CIK). And all the patients were drawn blood for the detection of CTCs.

2.2. Cell line

Carcinoma cell line SKBR-3 (breast) was used to estimate the sensitivity and specificity of the flow cytometry, which was maintained in RPMI 1640 plus 10% fetal calf serum.

2.3. Antibodies

Antibodies, which were used for multiparameter flow cytometry, were as follows: anti-CD45-PerCP, anti-CD44-APC (allophycocyanin, clone G44-26, catalog number 559942) and anti-CD24-FITC (fluorescein isothiocyanate, clone ML5, catalog number 555427) were from Becton Dickison Crop., USA. Ep-CAM (epithelial-cell adhesion molecule) and CK-8, CK-18, CK-19 (CK-8, CK-18, CK-19 phycoerythrin staining) were from Abcam, USA.

2.4. Preparation of samples

Every patient with breast cancer drew 20 ml blood for CTCs detection and three healthy volunteers. The blood of healthy volunteers was treated as negative control. About 5 ml blood was discarded to avoid contamination with skin cells as previously described [26]. We separated mononucleocytes from 15 ml blood by Ficoll-Paque (Haoyang Biological Production Limited Company, Tianjin, China) for 20 min with $1800 \times g$ at 25°C . One half of the mononuclear cells was resuspended in phosphate-buffered saline (PBS) for multiparameter flow cytometry on the account of at least $2-3 \times 10^6$ cells for each sample and the other half was kept in Trizol reagent (Invitrogen, UK) at -70°C until RNA extraction for RT-PCR.

2.5. Flow cytometry

Mononucleocytes were enriched and washed twice with PBS and then labelled antibodies that target white cell antigens and epithelial cell antigens (CD45-, Ep-CAM⁺, CK-8, CK-18, CK-19⁺), kept in dark at 4°C for 30 min. We added 20 μl monoclonal antibodies for each sample. Cell pellets were resuspended in 250 μl PBS and enumerated by FACS Caliber™ (Becton Dickison Crop., USA) at last.

SKBR-3 breast cancer cells were used to evaluate the sensitivity of the flow cytometry (tumor cells recovery). Tumor cells were resuspended and counted. 1, 10, 50, 500 cells were spiked, respectively, into 7.5 ml of blood from healthy person. And then, it was processed as described as earlier and control the preparation for the same volume of the cell suspension and cells were counted to accurately estimate the number of cells spiked into the blood. The average number of Ep-CAM and CK-8, CK-18, CK-19 positive cells on FACS Caliber™ was used to calculate the cells recovery.

The mononuclear cells (MNCs) for FCM were incubated with monoclonal antibodies: anti-CD45-PerCP, anti-EPCAM-PE, anti-CD44-APC and anti-CD24-FITC. About 20 μ l of each antibody was needed, and the condition was 4°C for 30 min away from light. Then, the MNCs were washed two times. Finally, cells were resuspended with PBS and analyzed on FACS Calibur™ (BD Bio) using Cell Quest software (BD Bio).

2.6. RT-PCR

We extract RNA with 1 ml trizol from the mononucleocytes, which were separated from 7.5 ml blood and then kept at -70°C. Add 0.2 ml chloroform and then centrifuge the sample at $12,000 \times g$ for 15 min at 4°C. The intact RNA, which was contained in the supernatants, was removed into a new tube. RNA was dissolved in 10 μ l RNase-free water after precipitating RNA with isopropyl alcohol and washing with 75% ethanol. Then, RNA was transcribed to cDNA by a reverse transcriptase in a total 10 μ l RT reaction solution, which was contained of 2 μ l 5x Reverse Transcriptase Buffer, 1 μ l dNTP (10 mM each), 0.25 μ l RNase inhibitor (10 U), 1 μ l oligo (dT)₁₅ primer (25 pmol), 5.25 μ l RNase-free water containing RNA (>0.5 μ g), 0.5 μ l avian myeloblastosis virus (AMV) reverse transcriptase (5U) (TaKaRa, China). The resulting cDNA was subjected to PCR amplification. PCR was composed of 2 μ l cDNA, 10 μ l Mix, 2 μ l primer of EpCAM, 6 μ l H₂O in a total volume of 20 μ l. The primer of EpCAM was as follows: 5'-GGACCTGACAGTAAATGGGGAAC-3'; 5'-CTCTTCTTTCTGGAAATAACCAGCAC-3' [18]. GAPDH mRNA primer detail was as follows: 5'-TGCACCACCAACTGCTTAGC-3'; 5'-GGAGGCAGGGATGATGTTCT-3' which was designed by Primer 5.0. The reaction condition was 95°C for 2 min to activate Taq DNA polymerase and it finally elongated 72°C for 7 min with 35 cycles. We detected the PCR products by ethidium bromide staining on a 1% agarose gel.

2.7. Statistical analysis

Correlation, regression analysis and a Mann-Whitney rank sum test were performed on titration experiments. Chi-square test was used to compare across CTC groups. Overall survival was performed to describe the condition of CTCs and prognosis and was estimated by the Kaplan-Meier. Comparison of groups used the log-rank test. All statistical tests were two-sided, and *P* values <0.05 were considered statistically significant. Analyses above were performed by using the SPSS 13.0.

3. Results

3.1. Sensitivity and specificity

3.1.1. Sensitivity

It is demonstrated that the sensitivity of the method of CTCs detection by using multiparameter flow cytometry was 0.001%, or 10^{-5} , according to the serial dilutions test, which is shown in

Figure 1a–d. It was highly reproducible on recovery and linearity across three separate experiments (**Figure 1e**). The recovery of the tumor events was quite correlated with the tumor events expected based on the serial dilutions ($R^2 = 0.997$). The recovery of the tumor cells was not significantly different from the tumor cells expected according to the serial dilutions ($P > 0.6$, Mann-Whitney rank sum test).

3.1.2. Specificity

The detection of CTCs by multiparameter flow cytometry contributed a higher specificity compared with RT-PCR. We detected the expression of Ep-CAM in three typical advanced breast cancer (ABC) patient (CTCs ≥ 5) and three limited breast cancer (LBC) patients (CTCs < 5). The result showed that the expression of Ep-CAM was positive for both patients with ABC and LBC as shown in **Figure 2**. It was hard to distinguish ABC with LBC by RT-PCR, while the flow cytometry could distinguish them obviously and quantitatively.

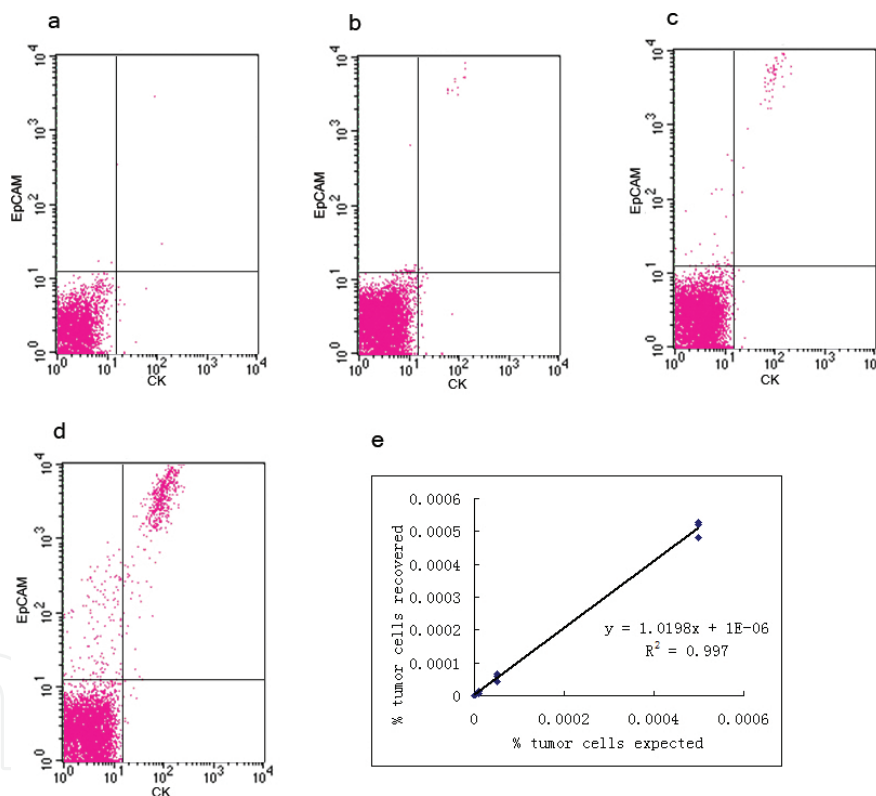


Figure 1. The ability to detect human tumor cells SKBR-3 cells in normal blood by cytometry is titratable down to a sensitivity of 0.001%. Human tumor cells concentration was normalized adding to the leukocyte count, and serial dilutions (0.0001, 0.001%, 0.005%, 0.05%) using normal mononucleocytes as the diluent. Samples were lysed, incubated with anti-CD45-PerCP (20ul), EpCAM (20ul) and Cytokeratin8, 18, 19 (20ul) at 4°C for 30 minutes, and resuspended in 250ul PBS before multiparameter flow cytometric analysis. For samples with normal blood cells, up to 1000,000 total events were collected. Events that fell within up right region were counted as meeting the criteria for SKBR-3 tumor cells (CD45-EpCAM+CK+). Representative SKBR-3 cells are shown for (a) 0.0001% (b) 0.001% (c) 0.005%, (d) 0.05%. (e) Correlation and regression analysis of recovered versus expected number of positive tumor events at dilutions significant at the 0.01 level (2-tailed, $R^2 = 0.997$, three separate experiments.). The percentage of tumor cells recovered was not significantly different from the percentage of tumor cells expected based on the serial dilutions ($P > 0.6$, Mann-Whitney rank sum test).

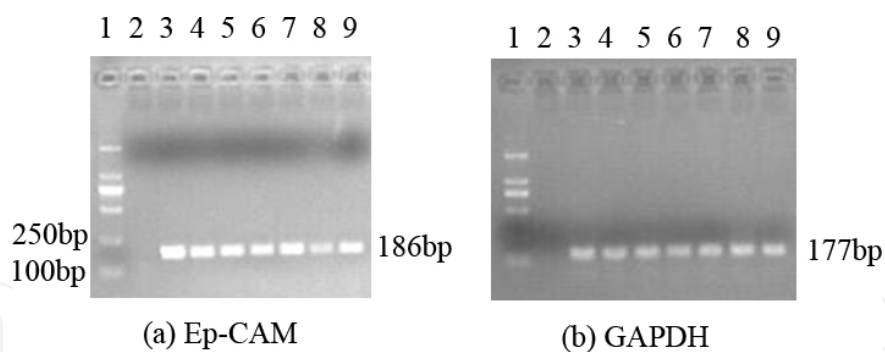


Figure 2. RT-PCR assay for EpCAM mRNA (a) and GAPDH mRNA (b). 1. DNA ladder, 2. negative control (H₂O), 3. positive control (SKBR-3 cells), 4.5.6. ABC samples, 7.8.9. LBC samples. All were positive. Size of EpCAM and GAPDH is 186bp and 177bp, respectively.

Patient no.	EPCAM (10 ⁻⁵)		CD44	
	CTC <50	CTC ≥50	CTSC negative	CTSC positive
1	24.41	97.66	0.38	0.41
2	2.99	72.99	0.36	0.62
3	4.96	48.81	0.48	0.96
4	3.29	1.66	0.92	0.82
5	1.19	3.41	0.75	0.63
6	2.99	24.41	0.66	0.46
7	2.28	24.39	1.04	0.84
8	6.31	48.85	0.44	0.57
9	5.17	39.39	4.20	0.17
10	48.83	5.50	1.01	0.34
11	69.05	48.88	1.17	0.41
12	30.27	2.81	0.84	1.43
13	4.38	11.08	0.06	3.39
14	41.92	6.10	0.63	0.99
15	10.05	5.98	0.58	0.49
16	75.56		0.43	0.47
17	2.62		0.50	
18	29.24			
Control	0.14 ± 0.02	0.59 ± 0.10		

Table 2. Analysis of EPCAM and CD44 gene expression in patients with BC.

We also compared breast cancer (BC) patients with healthy volunteers by QRT-PCR. The expression of Ep-CAM was increased statistically higher in patients with BC (24.29 ± 44.10 vs. $0.14 \pm 0.02 \times 10^{-5}$, $P = 0.000$) than in healthy volunteers, which was shown in **Table 2** and it was calculated by $2^{-\Delta t}$ method. However, there were no obvious differences between BC and health on the expression of CD44. Therefore, we confirmed that Ep-CAM and CD44 cannot be identified by QRT-PCR but can be identified by multiparameter flow cytometry.

3.2. Patient characteristics

Forty-five patients were identified and included in this analysis (detail is shown in **Table 1**); 27 (60.0%) patients had CTCs levels <5 and 18 (40.0%) patients had CTCs levels ≥ 5 , respectively. The age of all the 45 patients ranged from 32 to 74, while the median age of CTCs levels <5 group was 49 years and the median age of CTCs ≥ 5 was 51 years. There were 25 (55.6%) patients in metastasis including 15 (33.3%) patients had CTCs ≥ 5 . At the same time, there were 20 (44.4%) patients with no metastasis including 17 patients had CTCs <5 . It showed that there was statistically significantly differences ($P = 0.002$) on CTCs level between the metastasis group and no metastasis group by chi-square test analyses. And the statistical differences of CTCs level also exist in different TNM stage ($P = 0.033$).

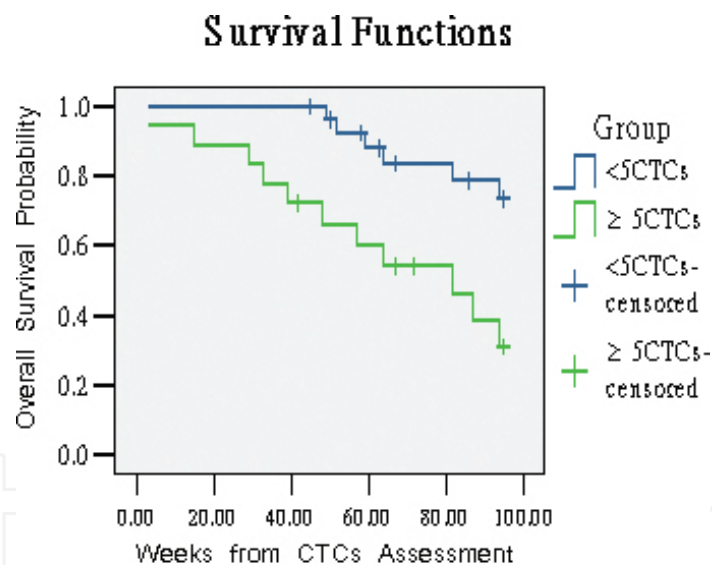


Figure 3. Kaplan-Meier Plots of overall survival are shown for all patients during the follow-up. 27 patients in CTCs <5 group and the median survival is estimated to be 95 weeks. 18 patients in CTCs ≥ 5 group and the median survival is 65.5 weeks. P value is 2-tailed. Logrank indicates the P value, $p = 0.004$.

3.3. Survival analysis

There were 17 (37.8%) patients who died during the follow-up period including 11 (24.5%) patients in CTCs ≥ 5 group. The median survival among CTCs <5 group was 95 weeks (standard deviation, 18.67 weeks) and the median survival among CTCs ≥ 5 was 65.5 weeks (standard deviation, 30.0 weeks). Axillary lymph node dissection (ALND) had correlation with CTCs

level ($P=0.143$) and 45.7% ALND patients got CTCs ≥ 5 . During the follow-up, 11 (24.4%) patients got lost contact with seven (15.6%) in < 5 CTCs and four (8.9%) in \geq five CTCs group. The results of the patients survival are shown in **Figure 3** by Kaplan-Meier with logrank $P=0.004$ and Breslow $P=0.003$, which confirm that the survival of CTCs < 5 and CTCs ≥ 5 group were different statistically. And the overall survival (OS) of CTCs < 5 group was higher than CTCs ≥ 5 group.

Variables	b_j	S_{b_j}	P	$expb_j$	95.0% CI for $expb_j$	
					Lower	Upper
Age	0.101	0.031	0.001	1.107	1.042	1.176
CTCs	1.204	0.589	0.041	3.333	1.050	10.581
ALND	-1.813	1.111	0.103	0.163	0.018	1.439
Diameter	1.029	0.822	0.211	2.799	.558	14.025
Metastasis	2.825	0.910	0.002	16.855	2.834	100.237

Table 3. Cox regression analysis results.

In addition, we found that the prognosis of patients with breast cancer was statistical significant in CTCs level ($P = 0.041$), age ($P = 0.001$) and metastasis ($P = 0.002$) base on the Cox regression analysis for the follow-up in 45 patients as shown in **Table 3**.

3.4. Circulating tumor stem cells

We also analyzed the expression of CTSCs and the characteristic of clinical data was shown in **Table 4**. There were 21 patients had at least one CTSCs expression among 45 patients with breast cancer. The CTSCs level in different TNM stages was statistically different ($P = 0.020$). It was obvious that stage III and IV patients contributed more CTSCs expression than stage I and II.

Patient characteristics	Number (%)	CTC (%)		P -value	CTSC (%)		P -value
		<50	≥ 50		Negative	Positive	
Age (years)				0.247			0.168
≤ 45	24 (53.3)	12 (50)	12 (50)		10 (41.7)	14 (58.3)	
>45	21 (46.7)	15 (71.4)	6 (28.6)		14 (66.7)	7 (33.3)	
TNM stage				0.0272*			0.0202*
0	4 (8.9)	4 (100.0)	0 (0.0)		4 (100.0)	0 (0.0)	
I	6 (13.3)	6 (100.0)	0 (0.0)		4 (66.7)	2 (33.3)	
II	14 (31.1)	8 (57.1)	6 (42.9)		10 (71.4)	4 (28.6)	
III	19 (42.2)	9 (47.4)	10 (52.6)		6 (31.6)	13 (68.4)	
IV	2 (4.4)	0 (0.0)	2 (100.0)		0 (0.0)	2 (100.0)	

Patient characteristics	Number (%)	CTC (%)		P-value	CTSC (%)		P-value
		<50	≥50		Negative	Positive	
Tumor size				0.155			0.165
Tis	4 (8.9)	4 (100.0)	0 (0.0)		4 (100.0)	0 (0.0)	
T1	10 (22.2)	7 (70.0)	3 (30.0)		7 (70.0)	4 (30.0)	
T2	20 (44.4)	12 (60.0)	8 (40.0)		8 (45.0)	11 (55.0)	
T3	5 (11.1)	1 (20.0)	4 (80.0)		2 (40.0)	3 (60.0)	
T4	6 (13.3)	3 (50.0)	3 (50.0)		2 (33.3)	4 (66.7)	
RLNM				0.075			0.0012*
0	24 (53.3)	16 (66.7)	8 (33.3)		19 (79.2)	5 (20.8)	
1-3	9 (20.0)	7 (77.8)	2 (22.2)		3 (33.3)	6 (66.7)	
>3	12 (26.7)	4 (33.3)	8 (66.7)		2 (16.7)	10 (83.3)	
Clinical pathology				0.264			0.098
CIS	4 (8.9)	4 (100.0)	0 (0.0)		4 (100.0)	0 (0.0)	
Infiltrating duct	36 (80.0)	21 (58.3)	15 (41.7)		16 (44.4)	20 (55.6)	
Mucous	2 (4.4)	1 (50.0)	1 (50.0)		2 (100.0)	0 (0.0)	
Infiltrating lobular	1 (2.2)	1 (100.0)	0 (0.0)		1 (100.0)	0 (0.0)	
Medullar	1 (2.2)	0 (0.0)	1 (100.0)		1 (100.0)	0 (0.0)	
Papillary	1 (2.2)	0 (0.0)	1 (100.0)		0 (0.0)	1 (100.0)	
Histology stage ^a				0.002*			0.919
I	4 (10.5)	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)	
II	17 (44.7)	14 (82.4)	3 (17.6)		8 (47.1)	9 (52.9)	
III	17 (44.7)	4 (23.5)	13 (76.5)		7 (41.2)	10 (58.8)	
ER				0.0182*			0.482
-	13 (28.9)	4 (30.8)	9 (69.2)		8 (61.5)	5 (38.5)	
+	32 (71.1)	23 (71.9)	9 (28.1)		16 (50.0)	16 (50.0)	
PR				0.0052*			0.771
-	16 (35.6)	5 (31.3)	11 (68.7)		9 (56.3)	7 (43.7)	
+	29 (64.4)	22 (75.9)	7 (24.1)		15 (51.7)	14 (48.3)	
Her-2				0.405			0.262
1+	14 (31.1)	10 (71.4)	4 (28.6)		10 (71.4)	4 (28.6)	
2+	18 (40.0)	11 (61.1)	7 (38.9)		8 (44.4)	10 (55.6)	
3+	13 (28.9)	6 (46.2)	7 (53.8)		6 (46.2)	7 (53.8)	

CIS, carcinoma *in situ*.

^aThere are missing values.

* $P < 0.05$.

Table 4. Patient characteristics in different CTC and CTSC levels.

The CTSC positive was also related with RLNM status (RLNM 0, 20.8%; RLNM 1–2, 66.7%; RLNM >3, 83.3%) and the *P*-value was 0.001. There were no statistical differences in regard to age, diameter of tumor, clinical pathology, histology stage, ER status, PR status and Her-2 status in different CTSC groups.

3.5. Percentages of CD45-cells, CTCs, CTSCs and their clinical relevance

The expression of CD45, CTCs and CTSCs was further explored in different BC groups (Table 5). The expression of CTSCs on CD45-C showed a rising tendency in different TNM stage (*P* = 0.034) and also in different RLNM status (*P* = 0.001).

Patient characteristics	CD45/MNC (%)	CTC/CD45 ⁺ (%)	CTSC/CD45 ⁺ (%)	CTSC/CTC (%)
TNM stage	a	a	*c	c
0	2.01 ± 1.08	0.11 ± 0.12	0.00 ± 0.00	0.00 ± 0.00
I	1.29 ± 0.92	0.24 ± 0.25	0.03 ± 0.05	5.38 ± 11.46
II	2.72 ± 5.26	0.42 ± 0.51	0.06 ± 0.14	3.72 ± 7.63
III	2.52 ± 2.22	0.48 ± 0.69	0.10 ± 0.09	2.71 ± 3.78
IV	1.94 ± 0.88	1.00 ± 0.53	0.29 ± 0.35	5.45 ± 2.05
RLNM	a	c	*c	a
0	2.43 ± 4.01	0.28 ± 0.28	0.02 ± 0.05	2.49 ± 6.30
1–3	1.99 ± 1.66	0.25 ± 0.21	0.14 ± 0.17	4.77 ± 8.89
≥4	2.46 ± 2.59	0.83 ± 0.90	0.14 ± 0.15	3.67 ± 4.05
Clinical pathology	c	*a	a	a
Carcinoma <i>in situ</i>	2.01 ± 1.08	0.12 ± 0.12	0.00 ± 0.00	0.00 ± 0.00
Infiltrating duct	2.00 ± 1.79	0.37 ± 0.38	0.09 ± 0.14	4.07 ± 6.86
Mucous	11.57 ± 13.04	0.21 ± 0.30	0.00 ± 0.00	0.00 ± 0.00
Infiltrating lobular	1.13 ± 0.00	0.24 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Medullar	0.84 ± 0.00	1.34 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Papillary	0.47 ± 0.00	3.14 ± 0.00	0.13 ± 0.00	0.42 ± 0.00
Histology stage	a	a	a	c
I	2.56 ± 1.78	0.18 ± 0.16	0.03 ± 0.04	7.43 ± 14.10
II	1.38 ± 0.85	0.34 ± 0.41	0.10 ± 0.14	4.32 ± 7.15
III	2.33 ± 2.29	0.66 ± 0.76	0.10 ± 0.14	2.56 ± 3.31
ER	b	b	b	b
-	1.55 ± 0.78	0.52 ± 0.41	0.08 ± 0.16	2.10 ± 3.03
+	2.67 ± 3.80	0.38 ± 0.61	0.07 ± 0.11	3.73 ± 7.24
PR	b	b	b	b

Patient characteristics	CD45/MNC (%)	CTC/CD45 ⁻ (%)	CTSC/CD45 ⁻ (%)	CTSC/CTC (%)
TNM stage	a	a	*c	c
-	2.09 ± 2.15	0.51 ± 0.42	0.08 ± 0.15	2.37 ± 3.37
+	2.49 ± 3.76	0.37 ± 0.63	0.07 ± 0.11	3.76 ± 7.49
Her-2	a	a	c	a
1+	2.93 ± 5.20	0.33 ± 0.47	0.03 ± 0.05	3.21 ± 7.63
2+	2.45 ± 2.32	0.36 ± 0.35	0.09 ± 0.14	1.68 ± 2.80
3+	1.57 ± 0.99	0.61 ± 0.83	0.11 ± 0.15	5.51 ± 7.96

* $P < 0.05$.

^aANOVA test.

^bStudent's *t*-test.

^cKruskal-Wallis test.

Table 5. Percentage of CD45⁻ cells, CTC and CTSC, and their clinical relevance.

The percentages of CTCs on CD45-C with TNM and histology stage increasing. In addition, we found that the percentage of CTCs on CD45-C in ER⁻ and PR⁻ groups was higher than that in ER⁺ and PR⁺ groups. And so was on CTSCs. Above all, we found the relationship between the percentage of CTC on CD45-C and clinical pathology. Then, it was good for evaluate TNM stage and RLNM status according to the percentage of CTSC on CD45-C.

Therefore, multiparameter flow cytometry technique is capable enough to identify patients with breast cancer and assess the progression of disease.

4. Discussion

4.1. The significances of CTCs in breast cancer

The detection of CTCs by using multiparameter flow cytometry relied on the epithelial-specific marker, which was expressed on epithelial cells but not on leukocytes [2, 27, 28]. Some of the CTCs that had higher metastatic potential may lose the expression of epithelial-specific markers during the migration process [29–31]. We also target another epithelial-specific marker—Ep-CAM (epithelial-cell adhesion molecule) to avoid possible false negative [32]. In order to detect the tumor cells that come from epithelium tissue, a monoclonal antibody directed against CD45 for negative selection of leukocytes [9–11]. Therefore, we targeted the dual-positive cells (CD45-EpCAM⁺CK⁺) as a surrogate marker for CTCs. And the serial dilution test was demonstrated to confirm the sensitivity of the assay by adding SKBR-3 into healthy sample. At the same time, we also verified the higher specificity of multiparameter flow cytometry by comparing with RT-PCR, although RT-PCR had a higher sensitivity [10, 11, 33, 34]. However, the detection of CTCs by nucleic acid techniques may overestimate the sensitivity, which resulted from the membrane fragments or nucleic acid of markers because of the crack of tumor cells in circle. CTC detection should be performed on cell level. On the other

hand, we came out the result that RT-PCR could not identify the ABC patient (CTCs ≥ 5) from LBC patients (CTCs < 5) because of the high sensitivity of RT-PCR technology. We chose the multiparameter flow cytometry as the way to detect CTCs under considering the balance of sensitivity and specificity. It was important to discard the first few milliliters of sampled blood to make sure that there was no false positive because of the epithelial cell fell off from the skin when punctured.

Some researchers had tried the immunomagnetic combining flow cytometry technique to detect CTCs, which had a higher false negative losing amount of target cells. This technique cost expensive and not brief enough to apply in clinical.

It was common to meet the problem in clinical that it was hard to distinguish the tumor from inflammation by imaging, while we found that there were no more than two CTCs in stage I. Budd et al. [35] proposed that it was more accurately to evaluate the development of disease by CTCs detection than imaging. Both tissue biopsy and marrow biopsy were traumatic, but CTCs detection by multiparameter flow cytometry was atraumatic that can be achieved in clinical. Above all, multiparameter flow cytometry was the appropriate technique for the detection of CTCs to monitor the progression of breast cancer.

We demonstrated that the retrospective study to confirm the CTCs detection by multiparameter flow cytometry technique was a value method to apply in clinical. It suggested the patients who had CTCs ≥ 5 prompting a poorer median overall survival (65.5 weeks vs. 95 weeks; $P < 0.05$). We also found that the prognosis of the breast cancer was related with CTCs level, age and metastasis but not the clinical pathology and diameter of tumor. Cristofanilli et al. [36] also proposed that the patients with ≥ 5 CTCs/7.5 ml common had poorer prognosis compared with patients with < 5 CTCs. CTCs detection by multiparameter flow cytometry should be a significant method for the evaluation of development and prognosis of cancer, and it also helped to estimate the treatment of target therapy for patients.

4.2. The significances of CTSCs in breast cancer

We chose CD44⁺CD24⁻ as an excellent marker in CTSCs identify. CD44⁺CD24⁻ had been considered as the marker of CSC [17, 37]. While CD133 was much more restricted in expression compared with CD44 that was the reason we did not chose CD133 as the CTSCs marker [38–40]. ALDH1 was another maker to identify CSC from BC. Ginestier et al. reported that the expression of ALDH1 in normal and breast cancer was 3–10%, while expression of CD44⁺CD24⁻ was 31% in contrast [41, 42]. In summary, CD44⁺CD24⁻ was an excellent maker in CSC identify.

CTCs may exist after mastectomy and chemotherapy; even there was no clinical manifestation of breast cancer. It was explained as the theory of “dormancy” in tumor cells [43]. And it was a part of tumor stem cells. Once the balance between proliferation and apoptosis was destroyed by some inducement, the disease progressed. The immune system and angiogenesis was reported, which were correlated closely with tumor cell dormancy [43, 44]. When the tumor stem cells fell off the primary tumor, they came into the peripheral circle and became the circulating tumor stem cells (CTSCs).

We successfully confirmed that the existence of CTSCs and also reveal the relationship between CTSCs level and different TNM stages. The correlation of CTSCs and clinical pathologic features remains unclear before. Previous study had reported that the CD44⁺CD24⁻ cancer stem cells was not correlated with clinical features such as lymph node status, tumor size, histology grade, ER, and PR, or HER-2 [45, 46].

It was reported that the patients who had high expression of CD44⁺CD24⁻ tumor stem cells were related with distant metastasis, particularly osseous [45]. And we found the expression of CTSCs was quite related with RLNM status. It was a novel way for treatment targeting at CTSCs to prevent metastasis and to evaluate the prognosis.

Cancer stem cells (CSCs) had been confirmed existed in many kinds of epithelial malignancy [47]. And the CSCs were considered as a subpopulation of tumor cells [48]. The mutation of normal stem cells resulted in the genesis of CSCs, which had been demonstrated by Cariati and Purushotham [49]. Therefore, CTSCs were suggested that it was generated not only from CTCs but also from normal stem cells. Further researches needed to be performed to confirm it.

Detection of CTCs and CTSCs by using multiparameter flow cytometry was considered as an effective technique on monitoring disease development and evaluating prognosis [50, 51]. Using multiparameter flow cytometry to detect CTC and CTSC in peripheral blood may provide new opportunities for the early diagnosis of invasive breast cancer, guide us to select optimal therapeutic regimens and help us to predict the prognosis. Moreover, we wish to isolate the CTSCs that have the marker of CD44⁺CD24⁻ESA⁻ expressing. Further researches on CTSCs needed to be demonstrated and we have established a firm basis for following research.

5. Conclusion

Detection of CTCs by using multiparameter flow cytometry was effective and it has the potential to be a valuable method for both prognosis assessment and cancer research in breast cancer.

6. Looking forward

There is no doubt that CTCs would become an important test in clinical in near future. The challenge of the achievement contains the heterogeneity of CTCs and big database of clinical on CTCs. The heterogeneity of CTCs reflected in different tumor related proteins and morphology. However, researchers have pay more and more interesting on EMT phenotype that is common in all kinds of tumors. It will make CTCs detection accessible by decipherment of conundrums underlying EMT phenotype.

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