Pathology & Oncology Research
https://doi.org/10.1007/s12253-020-00847-7

ORIGINAL ARTICLE

In Vivo Detection of CTC and CTC Plakoglobin Status Helps Predict Prognosis in Patients with Metastatic Breast Cancer

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Received: 9 July 2019 / Accepted: 10 June 2020

Abstract
This study aims to detect the prognostic value of circulating tumour cell (CTC) in patients with metastatic breast cancer. In this study, 38 patients with metastatic breast cancer were enrolled. The *in vivo* CellCollector® method was used to detect the number of CTC in patients. Single CTC and CTC clusters were counted, and the expression of plakoglobin was also analysed. At baseline, 73.7% (28/38) of the patients were positive for ≥ 1 CTC (range, 1–14 cells). No CTC-like events were observed in the control group. Among the CTC-positive patients, 21.4% (6/28) of patients had CTC clusters, and 42.9% (12/28) of patients had plakoglobin-positive CTC. After chemotherapy, 48.6% (17/35) of the patients were positive for ≥ 1 CTC (range, 1–3 cells), of which 3 patients had CTC clusters, and 35.3% (6/17) had plakoglobin-positive CTC. Additionally, we found that the number of CTC clusters in plakoglobin-positive patients was much greater than that in plakoglobin-negative patients, and the number of CTC was associated with the number of sites of metastases. We also found that patients with ≥ 3 CTC at baseline had shorter progression-free survival (PFS) and overall survival (OS), and pre-chemotherapy CTC detection was associated with PFS ($P = 0.0001$) and OS ($P = 0.0091$). CTC plakoglobin expression was associated with PFS ($P = 0.02$) but not OS ($P = 0.22$). CTC collected by the *in vivo* CellCollector method in Chinese patients with metastatic breast cancer have prognostic significance. CTC plakoglobin expression may be associated with CTC clusters, and more in-depth studies are needed.

Keywords Circulating tumour cells · CellCollector · Metastatic breast cancer · Plakoglobin · Progression-free survival · Overall survival

Background

Breast cancer is the most common cancer and the second highest cause of cancer mortality in females. It accounts for 30% of all new cancer diagnoses in women [1]. Although patients with breast cancer account for nearly half of all cancer survivors [2], metastatic breast cancer (MBC) is still considered to be an incurable disease and a main cause of cancer-specific death [3]. For MBC patients, the main purpose of treatment is to control the disease, relieve symptoms, and prolong survival time. Therefore, efficacy monitoring is of great importance, and at present, clinical efficacy is mainly evaluated by imaging methods. Meanwhile, it is self-evident that prognostic evaluation is critical for clinical treatments in MBC patients, but unfortunately, there is still a lack of noteworthy methods for predicting the prognosis of MBC patients.

Circulating tumour cells (CTCs) are those tumour cells detached from tumour tissues, circulating in the bloodstream and reaching distant sites after extravasation. The time that CTC reside in the blood has been a controversial issue, with estimates ranging from hours to days [4]. One study indicated that the half-life for CTC was 1–2 h [5]. However, CTC can be detected in dormant patients 8–22 years post mastectomy. Studies have shown that escaped tumour cells enter the bloodstream through blood vessels deep into dense tumours [6]; for example, breast cancer cells that leave the primary tumour by blood vessels will be carried by the blood flow first through the heart and then to the capillary beds of the lung. Some cancer cells might reside in the lung, while others pass through it and enter the systemic arterial system, where they are

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Published online: 18 June 2020

transported to remote organs, such as bone and the liver [7]. Though only a few tumour cells that escape into the circulatory system can survive and lead to organ metastasis, CTC is still promising biomarker that have already shown prognostic relevance in several kinds of metastatic cancers [8–13]. The 8th edition of the AJCC cancer staging manual also mentions that CTC is associated with poor prognosis in patients [14].

There are challenges with CTC capture and identification because there is a lack of effective methods for CTC detection. Until recently, there have been many *in vitro* techniques, such as CellSearch, for detecting CTC. Due to the limited volume of blood analysed, the positive rate of detection is very low, which restricts its clinical application [15]. To overcome the limitations of small sample volumes, the *in vivo* CellCollector® method was developed. It offers the possibility of capturing and identifying CTC in transit within the circulation of patients, and the identification of CTC is based on the phenotypic differences between CTC of epithelial origin and cells of haematopoietic origin using markers specific to each population [16, 17].

Interestingly, the presence of CTC clusters in the bloodstream has been reported to be associated with clinically important factors and the response to chemotherapy [18, 19]. Plakoglobin (also known as γ-catenin) is an important component of catenin (a junctional complex structure for cell–cell adhesion) and adherence junctions [20]. Studies have indicated that persistent CTC clusters are associated with adverse clinical outcomes. It has also been indicated that CTC clusters consistently have a higher expression of plakoglobin than single CTC [18].

In this study, we aimed to evaluate the detection rate of the novel *in vivo* CTC detection technology CellCollector in MBC patients and the expression of plakoglobin in CTC. Furthermore, we analysed the correlation between CTC and clinical parameters, as well as CTC counts and the expression of CTC plakoglobin, to predict the prognosis of MBC patients.

Methods

Study Design

This was a single-centre population-based screening program for MBC patients from May 2015 to June 2017 in Hunan Cancer Hospital. Thirty-eight MBC patients were enrolled in this study. The diagnosis of breast cancer in all patients was confirmed by histological analysis. CTC was analysed by using immunofluorescence staining. In addition, 25 healthy subjects randomly selected from the healthy volunteers underwent CTC detection. Approval was obtained from the Hunan Cancer Hospital ethics committee (Changsha, Hunan, China), and all participants provided written informed consent.

In Vivo Application of the CellCollector

CellCollector (GILUPI GmbH, Potsdam, Germany) is comprised of stainless steel wire widely used in medicine with a 20 mm functional area that is plated with a thick gold layer deposited on the device by galvanization. Antibodies to the epithelial cell surface antigen EpCAM are attached to a polycarboxylate/hydrogel that is coated on the gold-plated layer. CellCollector captures target cells expressing EpCAM antigen on the cell surface.

CellCollector is inserted into the peripheral blood of the vein through a 20G peripheral venous catheter and stays there for 30 min. The device is exposed to approximately 1500 ml of blood. During this time, CTC from the blood can bind to anti-EpCAM. The correct length of insertion is indicated by a mark on the distal part of the wire, which is not inserted into the cannula.

CTC Identification and Plakoglobin Expression Analysis

After finishing the CellCollector application, the wire is removed and washed, and the native CTCs isolated from the blood of the patient can be further analysed. Immunocytochemical staining was used to identify whether the cells were tumour cells or white blood cells. Cells attached to the wire were incubated with CK7, CK19, panCK (EXBIO Praha, clone A53-B/A2-Alexa488) and an APC-conjugated rabbit antibody against CD45 (Exbio, clone MEM-28-Alexa647). For CTC plakoglobin identification, PE was coated with plakoglobin antibody. The cells were counterstained with the nuclear dye Hoechst 33342 (Sigma, H6024). The intensity of the immunocytochemical staining of CTC was evaluated using an Axio Imager. CTC-positive cells were defined as CK-positive, CD45-negative nucleated cells. Cytokeratin-positive cells should have additional features, including a large cell body (diameter 10–50 μm), an irregular cell shape, a large irregularly shaped nucleus, and a high nuclear to cytoplasmic ratio. The cells were enumerated on each CellCollector by an operator blinded to the clinical information of the patients. CTC clusters were classified as cellular groupings that had more than two tumour cells banding together [18].

Statistical Analysis

Differences between the MBC group and the control group were compared by using Student's t test, Pearson's chi-square test, or Fisher's exact test as appropriate, and a P value < 0.05 was considered statistically significant. Survival was calculated

by the Kaplan-Meier method and compared by the log-rank test. Cox proportional hazards regression models were used to compute the univariate and multivariable analyses. All statistical manipulations were performed using SPSS 23.0 software, and all figures were constructed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA).

Results

Characteristics of the Study Individuals

A total of 38 MBC patients underwent CTC detection. In the MBC group, the median age was 52.5 years old, 44.74% (17/38) of the patients were ≤ 50 years old, and 60.54% (23/38) of the patients were menopausal. Moreover, the proportion of HER2-positive patients was 31.58% (12/38), while ER/PR-positive patients accounted for 65.79% (25/38). A total of 15.79% (6/38) of the patients had 1 site of metastasis, 26.32% (10/38) had 2 sites of metastases, and 57.89% (22/38) had at least 3 sites of metastases (Table 1). The median age of the 25 healthy subjects was 52 years old.

Overview of CellCollector and CTC Identification

CTC was detected and identified according to the method described above. CK-positive, nuclear-positive and CD45-negative cells were identified as CTC (Fig. 1a), while CK-negative, nuclear-positive and CD45-positive cells were identified as leukocytes. Tumor cells were identified as CK-positive (green) and CD45-negative (red) (Fig. 1b).

Table 1 Correlation between CTC and clinical features

	Total number of patients	CTC positive N (%)	*P*-value
Number of patients	38 (100)	28 (73.7)	
Age (years)			0.697
≤ 50	17 (44.74)	12 (70.59)	
>50	21 (55.26)	16 (76.19)	
HER2 status			0.900
Positive	12 (31.58)	9 (75.00)	
Negative	26 (68.42)	19 (73.08)	
ER/PR status			0.653
Positive	25 (65.79)	19 (76.00)	
Negative	13 (34.21)	9 (69.23)	
Menopausal status			0.428
Postmenopausal	23 (60.53)	18 (78.26)	
Premenopausal	15 (39.47)	10 (66.67)	
Number of metastases			0.001
One	6 (15.79)	2 (33.33)	
Two	10 (26.32)	5 (50.00)	
More	22 (57.89)	21 (95.45)	

CTC indicate Circulating Tumor Cell

CTC Counts and Correlations With Clinicopathological Characteristics

In total, 73.7% (28/38) of the patients were positive for ≥ 1 CTC (range, 1–14 cells) at baseline (pre-chemotherapy). No CTC-like events were observed in the control group (Fig. 2a-b). According to the number of sites of metastases (1, 2 and 3 or more), the patients were divided into three groups; the positive rates of CTC were 33.33% (2/6), 50% (5/10), and 95.45 (21/22), respectively. The results showed that CTC was associated with the number of sites of metastases ($P = 0.001$). However, we did not find correlations between CTC and age, menopausal status, HER2 expression, or ER/PR status (Table 1). Three patients were lost to follow-up, the remaining 35 patients underwent CTC detection after chemotherapy, and the positive rate was 48.6% (17/35, range, 1–3 cells) (Fig. 2a-b).

Plakoglobin Expression Analysis in CTC

Plakoglobin expression in CTC was analysed by immunocytochemical staining. SKBR3 and PBMC cell lines were used as positive and negative cells, respectively. Twelve patients were identified as having plakoglobin-positive CTC at baseline (Fig. 2c). The plakoglobin positive expression rate was 42.9%. Moreover, before chemotherapy, we found that 6 of the 28 CTC-positive patients had CTC clusters, of which 4 patients were plakoglobin positive. Among 22 patients without CTC clusters, 8 patients (36.36%) were plakoglobin positive. However, we did not find a significant correlation between plakoglobin expression and CTC status (CTC clusters or single CTC) ($P = 0.184$) (Fig. 2d). After chemotherapy, 3 patients still had CTC clusters, and 6 patients (35.3%) had ≥ 1 plakoglobin-positive CTC.

CTC and Survival

A total of 35 patients underwent CTC detection before and after chemotherapy. After chemotherapy, 4 patients had progressive disease (PD), of which 2 patients had unchanged CTC numbers, and 2 patients had increased CTC numbers. Nineteen patients had partial response (PR), of which 3 patients had unchanged CTC numbers, 2 patients had increased CTC numbers, and 14 patients had decreased CTC numbers. Twelve patients had stable disease (SD), of which 5 patients had unchanged CTC numbers, 1 patient had increased CTC numbers, and 6 patients had reduced CTC numbers (Fig. 3a). Pre-chemotherapy patients were stratified according to high (≥ 3 CTCs) or low (< 3 CTCs) pulmonary vein CTC groups. In

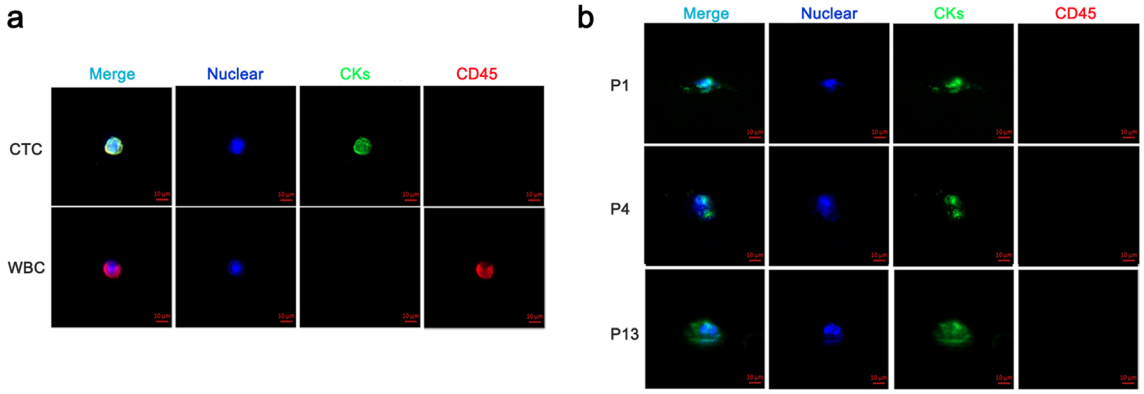

Fig. 1 CellCollector application and CTC identification. CTC identification standard by using IF staining (**a**). Representative CTC captured with CellCollector (**b**)

the log-rank test, pre-chemotherapy CTC detection was associated with PFS (hazard ratio, 7.486; 95% CI 2.538 to 22.08; $P = 0.0001$) and OS (hazard ratio, 12.54; 95% CI 1.874 to 83.94; $P = 0.0091$) (Fig. 3b-c). We also found that CTC plakoglobin expression was associated with PFS (hazard ratio, 3.146; 95% CI 1.206 to 8.205; $P = 0.02$), but we had not yet found a correlation with OS ($P = 0.22$) (Fig. 4).

The correlations between OS and the various clinicopathological factors are shown in Table 2. According to the univariate analysis, OS exhibited significant relationships with the number of sites of metastases ($P = 0.049$) and CTCs ($P = 0.014$). Accordingly, multivariable analysis had similar results, and OS exhibited significant relationships with the number of sites of metastases ($P = 0.020$) and CTC ($P = 0.029$).

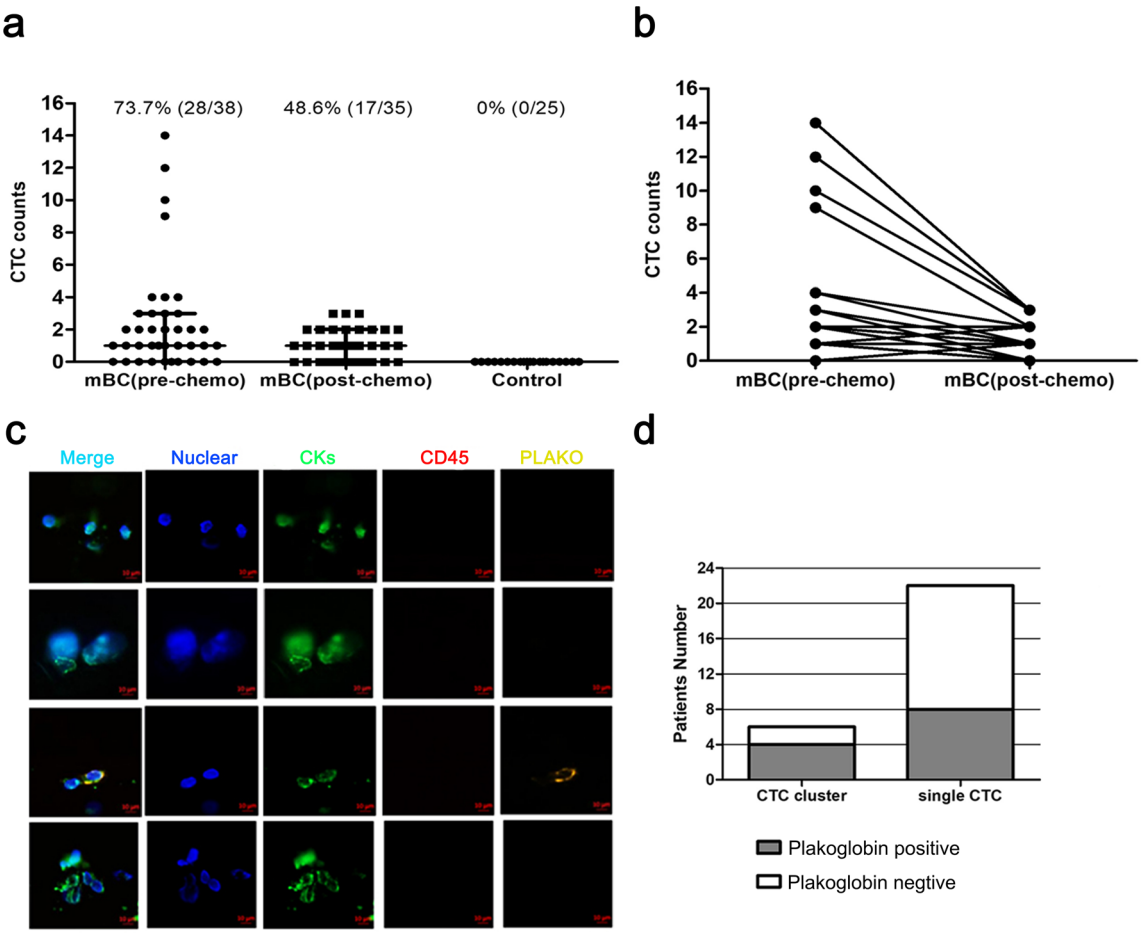

Fig. 2 CTC counts and plakoglobin expression analysis. CTC counts in MBC patients (first and second application) and the control group (**a**). CTC change analysis between the first and second capture numbers (**b**). CTC clusters in breast cancer patients (**c**). CTC cluster number analysis in plakoglobin-positive and -negative expression patients (**d**)

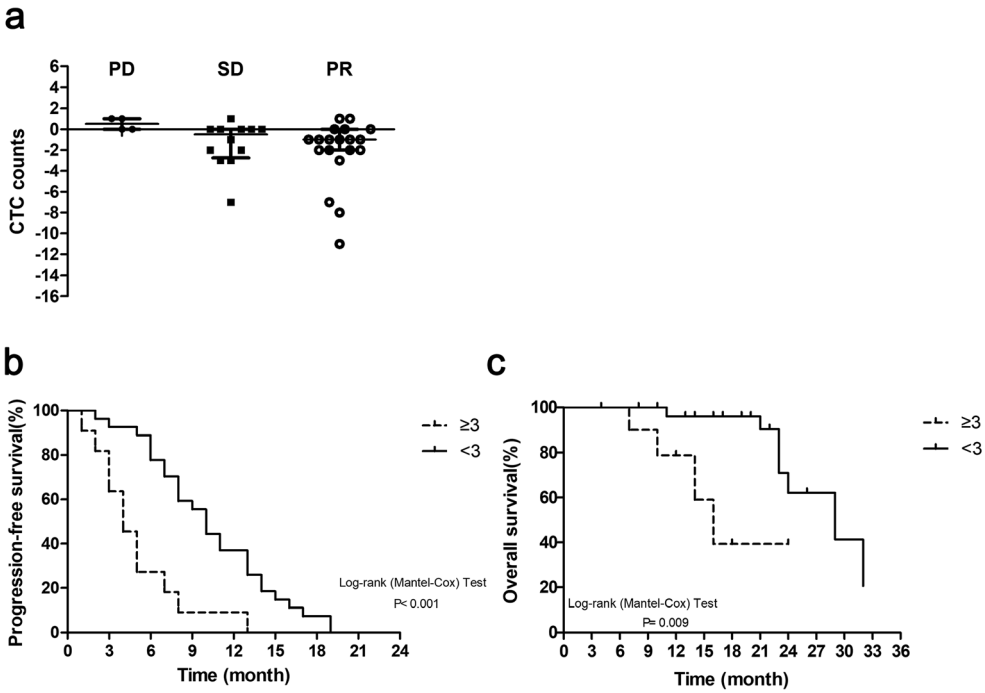

Fig. 3 CTC counts and prognostic analysis. Relationship between CTC number changes and treatment effect (**a**). Relationship between CTC and PFS (**b**). Relationship between CTC and OS (**c**)

Discussion

CellSearch, as the "gold standard" of CTC detection, has been approved by the Food and Drug Administration (FDA) and National Medical Products Administration (NMPA) for clinical use, and the prognostic value of CTC has been reported in some studies, suggesting that CTC 0 is an indicator that cancer cells circulating in the blood are no longer detectable, and this is an easily recognized outcome that is clinically meaningful to patients [21]. CellSearch is an *in vitro* blood collection method that mainly captures tumour cells with anti-EpCAM antibody specificity and then identifies CTC by EpCAM/CK/DAPI immunofluorescence staining. However, the CellSearch system still has some limitations, and the detection rate of CTC *in vitro* is relatively low, approximately 20% in patients with early breast cancer [22, 23]. The focus has now become developing a new platform with sufficient sensitivity to ensure that thesetests are carried out in a larger proportion of patients, as well as biological studies of the cells.

In this study, we performed a novel *in vivo* CTC capture assay and analysed the prognosis of Chinese MBC patients. There were no false-positive CTC findings in the randomly selected non-cancer group, and 73.7% (28/38) of the patients were positive for ≥1 CTC (range, 1–14 cells) at baseline. Therefore, CellCollector is a promising device for MBC patients. Here, we provide evidence that CTCs in Chinese MBC patients can be detected by CellCollector. As a novel *in vivo* CTC detection technology, CellCollector obtained CE certification in 2012, completed a registered clinical trial in China in 2016 and was approved by NMPA for metastatic breast cancer in 2017.

In our study, we found that CTC was correlated with the number of metastases. Moreover, the expression of plakoglobin was analysed in CTC. CTC clusters were also analysed in the plakoglobin-positive and -negative groups. Capturing CTC with an *in vivo* method may provide more CTC to analyse downstream protein expression [17]. Here, our study indicates that the numbers of CTC at baseline are

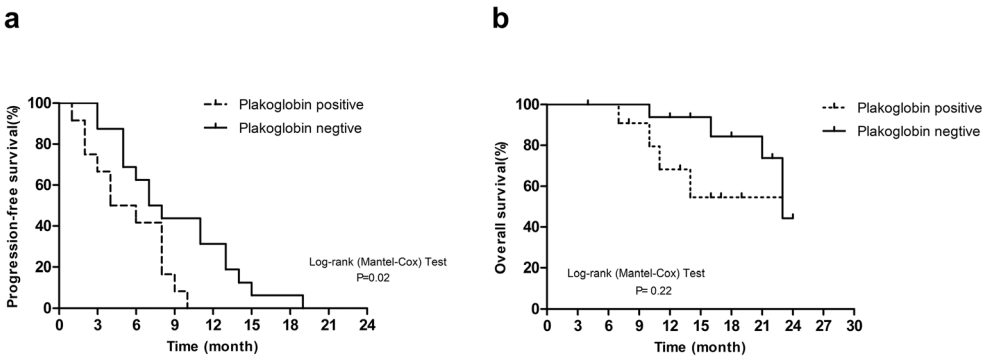

Fig. 4 CTC plakoglobin and prognostic analysis. Relationship between CTC plakoglobin and PFS (**a**). Relationship between CTC plakoglobin and OS (**b**)

Table 2 Univariate and Multivariable analyses of association among presence of CTC, clinicopathologic features, and overall survival in metastases breast cancer

Characteristic	Univariable analysis			Multivariable analysis		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age ≤ 50 vs. > 50	1.660	0.504–5.468	0.404	3.516	0.348–35.531	0.287
HER2 positive vs. negative	1.264	0.365–4.379	0.711	1.484	0.339–6.504	0.601
ERPR positive vs. negative	1.247	0.343–4.532	0.737	0.656	0.144–2.981	0.585
Premenopausal vs. postmenopausal	1.409	0.426–4.657	0.574	1.636	0.160-16.752	0.678
Number of metastases one or two vs. more	0.200	0.040–0.994	0.049	0.090	0.012–0.683	0.020
CTC ≥ 3 vs. < 3	0.179	0.045–0.707	0.014	0.180	0.039–0.838	0.029

HR indicates Hazard Ratio

CTC indicate circulating tumor cell

correlated with worse patient survival. Patients with ≥ 3 CTC had shorter PFS and OS, and pre-chemotherapy CTC detection was associated with PFS ($P = 0.022$) and OS ($P = 0.037$). The results were similar to those reported in other articles [24, 25], which showed that the number of CTC before treatment is a predictive factor for prognosis.

Previous studies have reported that the expression of plakoglobin was associated with the type of CTC, either alone or in clusters [18, 19]. In breast cancer patients, the rich presence of CTC clusters is associated with high levels of plakoglobin expression and poor prognosis, and plakoglobin expression is an independent prognostic factor for breast cancer patients [18]. In this study, CTC clusters had a higher proportion of plakoglobin expression than single CTC, which is consistent with the findings of previous studies, while patients with CTC plakoglobin expression had shorter survival. Previous studies have shown that CTC counts are associated with patient prognosis [24, 25]. In this study, we also found that patients with plakoglobin expression on CTC had shorter PFS. However, there were no differences in OS statistics, which may be due to the relatively small number of patients and short follow-up.

A recent study showed that stemness- and proliferation-related cells are hypomethylated on CTC clusters, and CTC cluster hypomethylation patterns are associated with poor prognosis in breast cancer [26]. One review of tumour metastasis suggests that there are two main mechanisms for promoting the metastasis of CTC clusters, namely, the formation of either plakoglobin or CD44-dependent CTC clusters to improve survival, metastatic properties, or the stemness of CTC clusters [27]. In our study, the expression of plakoglobin related to the CTC clusters was associated with a shorter PFS. Unfortunately, few CTC clusters were detected, with only 6 cases, so no further analysis was performed. In the future, the findings need validation in a larger, prospective study encompassing a broader patient population that is appropriately powered to verify the relationships between OS and CTC clusters and the expression of CTC plakoglobin.

Our first validation study of the medical wire produced encouraging results. Using this device, the CTC was collected in the vein for 30 min, and the amount of blood was approximately 1500 ml. This results in a higher detection rate. Moreover, another study showed that subsequent applications of the wire during the same visit are feasible and increase the capture rates for CTC at which time no significant differences between repeated CTC measurements were observable, indicating that the CTC results are reproducible [28]. However, there are still some limitations, and open issues need to be discussed. First, current anti-EpCAM antibodies used for capturing CTC have demonstrated proof-of-principle, but future generations of the device should include a mixture of different capturing antibodies to CTC. Second, this is a single-centre study with a relatively small sample size and short follow-up. We will conduct more in-depth studies and long-term follow-up.

Overall, this is a new attempt at the novel technology of collecting CTC for detecting distant metastasis in Chinese MBC patients. We hope our study will broaden research ideas and provide a new research method for the plakoglobin-involved metastasis processes.

Acknowledgements This study was supported by the Beijing Medical Award Foundation (YXJL-2016-0040-0001).

Compliance with Ethical Standards

Conflict of Interest All authors state that they have no conflicts of interest and declare that they have neither financial nor non-financial competing interests.

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