Diagnosis and Monitoring Value of Circulating Tumour Cells in Breast Cancer With an Optimised Microfluidic Device: A Retrospective Study

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Abstract

Background: Currently, there are various platforms for circulating tumor cells (CTCs) detection that need further improvement. Here we performed CTCs detection with a novel automatic CTCs isolation and enrichment platform, CytoBot® 2000.

Methods: We conducted a retrospective study with186 breast cancer patients and 20 healthy volunteers. Peripheral blood was obtained and the CTCs were detected immediately. The performance of CytoBot® 2000 was verified by spiking assay and clinical samples through the receiver operating characteristic (ROC) curve. The number of CTCs and CD45 positive CTCs were analyzed along with clinicopathologic features and clinical therapies. Additionally, a short-term follow-up review was presented for treatment monitoring with CTC.

Results: CTCs were discovered in 119 of 137 patients (86.86%) with malignant tumors and 31 of 49 patients (63.27%) with benign carcinoid, but not in healthy group. Statistical analysis revealed that CTCs could easily distinguish malignant breast cancer from benign carcinoid ($P < 0.0001$). Notably, CTCs were significantly correlated with tumour progression ($P = 0.0216$). When the cut-off value was 2, the ROC curve estimated the sensitivity and specificity of 74.5% and 81.2%, respectively. The number of CTC was significantly associated with tumour TNM stage ($P = 0.032$) and tumour size ($P = 0.0326$). In retrospective follow-up, the majority of patients (65.22%) exhibited a reduction in CTCs enumeration because of improved treatment. Additionally, the increased proportion of CD45 positive CTCs has found during treatment with different strategies.

Conclusion: The CTCs enumeration on CytoBot 2000® platform could accurately respond to the clinical diagnosis and treatment. CTCs and CD45 positive CTCs both have significance in clinical indication of cancer diagnosis and monitoring.

1 Background

Breast cancer (BC) has replaced lung cancer as the most widely diagnosed cancer globally. In 2020, nearly 2.3 million (11.7%) new cases of BC were diagnosed with 684,996 (6.9%) mortalities [1]. Although the mortality rate of BC has declined annually from 1998 to 2015, the decline has noticeably slowed down since 2017 [2, 3]. BC is already the fifth leading cause of death globally, accounting for 25% of all cancers in females and 1 in 6 deaths caused by cancers in females [4]. Improvements in survival for BC depend on accurate early diagnosis and advanced treatment.

The traditional clinical diagnosis of BC includes a comprehensive diagnosis assay, including screening techniques and biopsy. Despite the typical application of mammography (X-ray), ultrasounds, and magnetic resonance imaging (MRI) in clinical diagnostics, several limitations, such as high cost, radiation risks, and low sensitivity in the early stage, exist [5, 6]. The biopsy includes tissue biopsy and liquid biopsy. Tissue biopsy is the gold standard in the clinical practice for cancer diagnosis. Immunohistochemistry (IHC) and fluorescence in situ hybridization are the most common tissue biopsy

methods with higher accuracy. However, they still have the shortcomings of complex sampling, local trauma, and disease spread and are time-consuming. Peripheral blood (PB) containing various disease markers and cells is easily obtained and possesses great potential as the appropriate sample for early liquid diagnosis [7, 8]. Clinical tests using PB, including circulating free DNA (cfDNA) [9], microRNAs (miRNAs or miRs) [10], and disseminated tumour cells (DTCs) [11], have rapidly developed in recent years. These blood tests can often detect abnormally expressed genes and other metabolic substances and provide essential evidence for early diagnosis and surveillance of malignant diseases than traditional biopsies [12].

Circulating tumour cells (CTCs) were first discovered in 1969. CTCs detach from tumour tissue and enter into the circulatory system [13]. CTCs have received increasing attention from clinicians and medical researchers because these are significantly associated with cancer progression and prognosis [14, 15]. Some studies have revealed that CTC numbers in PB are associated with progression-free survival and overall survival of patients with BC [16, 17]. In the 8th edition cancer guidelines of the American Joint Committee on Cancer, CTCs are described as a prognostic factor to predict outcomes in patients with BC and serve as a promising indicator for early diagnosis of cancer [18]. To date, many methods for CTCs detection are available, such as PCR-based ctDNA detection [19], physical features (size)-based CTCs isolation, and typical immunomagnetic beads–based capture strategy [20]. CellSearch® immunomagnetic capture is the first CTCs detection system that approved by the US Food and Drug Administration (FDA); however, clinical trials have documented its poor capture efficiency of less than 40% [21–23], the sensitivity was 83.35% with the only specificity of 47.5% at a cut-off of 2 [24]. He et al. detected 15 patients with lung cancer by the CellCollector® system, and the positive rate of CTCs was only 53.33% [25]. Similarly, the Cyttel® system revealed a 63% positive rate in non-small cell lung cancer measurement [26]. These methods are still difficult to be applied in clinical practices because of the low cell capture rate and limitation on the downstream application, such as CTCs cell culture and the following sequencing [27].

In the current study, we presented a new, fully automated device for the isolation and enrichment of CTCs, CytoBot® 2000, developed by Holosensor Medical Technology Ltd. This platform is based on a microfluidics strategy combining the physical and immunological properties of CTCs to improve the capture rate of CTCs from PB greatly. To assess the performance of the platform, we performed retrospective research and enrolled 186 patients with BC. The correlation between CTCs and clinicopathologic features and the adjuvant analysis of the efficacy of treatments were discussed.

2 Material And Methods

2.1 Patients with BC and ethics

In the study, 186 patients with BC from October 2020 to August 2021, from Fudan University Shanghai Cancer Center and The First Affiliated Hospital of Soochow University, were systematically reviewed (Figure 1). 20 individuals of healthy (validated by comprehensive medical examination) recruited in this

work for control. The patients were classified into benign (intraductal papilloma carcinoid, fibroepithelial carcinoid, carcinoid adenoma) and malignant (ductal tumour) groups depending on their clinical diagnosis. The clinical pathological features of BC patients were present in Supplement Table 1. Inclusion criteria were as follows: a) 18–80 years old; b) patients with BC with biopsy and imaging examination; c) Eastern Cooperative Oncology Group (ECOG) ≤ 2; d) Planning for surgery, neoadjuvant therapy, radiotherapy, drugs targeting, or hormonotherapy; e) without other malignant tumours in past 5 years; f) signed consent forms and agreed with PB sampling and examination. Exclusion criteria were as follows: a) ECOG > 2; b) pregnancy or suckling period; c) enrolled in other clinical trials not suitable for the current study; d) undergoing or previously received cancer treatment in past 5 years; e) no evident diagnostic outcomes; f) history of infectious disease, hepatitis B, HIV, or syphilis; g) no pharmorubicin treatment in past 3 months, and h) with other concomitant malignancies.

Table 1
Circulating tumor cells detection and clinical relevance analysis.

Subjects	N	CTC positive	CTC detection rate (%)	Average CTC number (range)	*p* value
Healthy	20	0	0	0	**<0.0001**
Benign	49	31	63.27%	0.92 (0-4)	
Malignant	137	119	86.86%	3.23 (0-13)	
Cancer stage					
0	1	1	100.00%	1	
	29	25	86.21%	2.59 (0-5)	**0.0216**
	57	47	82.46%	2.81 (0-10)	
	43	40	93.02%	4.05 (0-13)	
	7	6	85.71%	4.71 (0-13)	
Tumor size					
T1	40	34	85.00%	2.50 (0-8)	**0.0349**
T2	71	60	84.51%	3.38 (0-11)	
T3	16	15	93.75%	3.44 (0-10)	
T4	9	9	100.00%	5.22 (1-13)	
Node stage					
N0	62	53	85.48%	2.56 (0-7)	**0.0103**
N1	31	26	83.87%	3.32 (0-10)	
N2	28	24	85.71%	3.68 (0-11)	
N3	16	16	100.00%	4.86 (1-13)	
Metastasis					
M0	130	113	86.92%	3.15 (0-13)	0.1279
M1	7	6	85.71%	4.71 (0-13)	
HER-2 state					
Positive	51	45	88.24%	2.84 (0-13)	**0.0201**
Negative	81	70	86.42%	3.94 (0-13)	
Histology grade					

Subjects	N	CTC positive	CTC detection rate (%)	Average CTC number (range)	*p* value
1	6	6	100.00%	3.50 (1-6)	0.8095
2	50	47	94.00%	2.96 (0-9)	
3	48	41	85.42%	3.15 (0-11)	

The human specimen sampling and examination in the current study have followed the Declaration of Helsinki, and the research was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (050432-4-1911D) and The First Affiliated Hospital of Soochow University (2021050). All the patients agreed and signed the informed consent forms.

2.2 Cell lines

Human BC cell lines, MCF7 and SK-BR-3, were purchased from American type culture collection, USA. Cells were cultured in a Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 10% fetal bovine serum (FBS, Gibco), streptomycin, and penicillin. BC cells were maintained at 37°C and 5% CO_2.

2.3 PB collection and preparation

PB from patients with BC was collected before surgery or treatment. We ensured that the blood samples were not less than 4 mL, stored in EDTA tubes (Becton, Dickinson and Company), and pre-treated within 6 h.

Before CTCs detection, PB mononuclear cells (PBMC) were isolated from PB. Briefly, 4 mL density gradient separation solution (Dakewe Biotech, Shenzhen) and a diluted blood sample (4 mL of PB with an equal volume of phosphate buffer, pH = 7.0, Biological Industries, Israel) were added sequentially in a sterile 15-mL centrifuge tube and centrifuged at 700 *g* for 20 min at room temperature. The PBMC phase was carefully pipetted into a new 15-mL centrifuge tube, washed twice with 5–10 mL PBS and centrifuged at 500 *g* for 5 min at 25°C.

2.4 Spiking assay

MCF7 and SK-BR-3 cells were digested by trypsin (0.25%, T4799, Sigma-Aldrich) and counted with a counter. To mimic clinical samples that collected from BC patients, 30-40 of the commercial cancer cells were spiked into 4 ml PB that collected from healthy individuals. Then, this mixture processed with procedure in 2.3 item of this section before performed CTC assay on CytoBot® 2000. The cell capture rate of this platform is calculated with: the number of captured cancer cells/the number of total spiked cancer cells, the data was shown with average of capture rates.

2.5 CTCs detection

CTCs were detected using CytoBot® 2000, a novel CTCs platform based on advanced techniques, including microfluidics and immunoenrichment. In brief, CTC capture chips are manufactured using a metal mesh with 15-μm diameter pores, gold-covered; polymers and capture antibodies (EpCam, ab223582, Abcam) were incubated onto the surface to form a capture chip with unique functionality. The PBMC was resuspended using PBS to make a volume of 300 μL and loaded into a cell unit containing a capture chip. CTCs captured on the chip were further identified using the CytoBot® 2000 system (Holosensor Medical Technology Ltd., Jiangsu). In summary, the PBMC solution was passed through the microfluidic channels at a specific flow whose velocity was driven by a suction pump. Background cells smaller than CTC, such as white blood cells, could pass through the chip. CTCs were intercepted and captured by the antibody on the chip.

Further, immunofluorescence staining was performed using Pan-cytokeratin (ab215838, Abcam, Cambridge), CD45 (ab8216, Abcam, Cambridge), and DAPI (D9542, Sigma, Louis Missouri), followed by in situ washing with buffer. The cell filter was disassembled and observed under a fluorescence microscope (RX50M, Sunny Optics) (Figure 2A). The captured CTCs were verified and counted with $CK^{+}CD45^{-}DAPI^{+}$.

2.6 Statistical analysis

All data were analysed using GraphPad Prism 7.0 (GraphPad Software, California) and SPSS 24 (IBM, Almonck). Chi-square and unpaired t-tests were used for continual variables. Multiple groups were compared using one-way ANOVA. The receiver operating characteristic (ROC) curve was used to assess the sensitivity and specificity of the CTCs test, and the best cut-off value was obtained using the Youden index (sensitivity + specificity − 1). $P < 0.05$ was considered significant, and $P < 0.01$ was considered extremely significant.

3 Results

3.1 BC cells detection and identification with CytoBot® 2000

To assess the efficiency of CTCs detection, the validation of performance was performed using cell suspension and spiking assay. The cell suspension was consisted with BC cells (50–60) and Jurkat cells (1 million). The cancer cells were captured on the chip and stained in situ. The biomarker of solid tumour cells, cytokeratin (CK), was used for CTCs detection. CD45, the unique marker on the leukocyte surface, was used for exclusion. The nucleus was stained using DAPI. Consistent with previous reports [28], MCF7 and SK-BR-3 were presented as $CK^{+}CD45^{-}DAPI^{+}$. As shown in Figure 2B, the BC cells were detected by immunofluorescence (IF) of CK and DAPI. Additionally, we discovered that the CytoBot® 2000 had a higher detection rate (more than 80%) and precision (shown as CV%) (Figure 2C). In spiking assay, 30-40 of cancer cells were added into 4 ml of PB which collected from healthy individuals. After enrichment by CytoBot® 2000, the cancer cells were captured and indicated in Fig. 2D. The performance of this device shown with capture rate at 73.12% (MCF7) and 76.70% (SK-BR-3) respectively (Fig. 2E).

3.2 CTCs detection and clinical significance

Further, we performed the detection of CTCs with 20 healthy individuals and 186 patients with BC from the laboratory centre of the 2 hospitals. Overall, 137 patients with malignant BC were diagnosed through tissue biopsy and imaging technologies, whereas the remaining 49 cases were filed to benign groups. In the current study, CTC was not detected in the healthy normal but successfully detected in 119 of 137 malignant patients (86.86%) with an average CTCs count of 3.23 per 4 mL of blood, which was significantly higher than that in the benign group ($P < 0.0001$). In contrast, the assay detected CTCs in 31 of 49 patients (63.27%) with an average CTCs count of 0.92 per 4 mL of blood sample (Table 1, Figure 3A). In immunofluorescence images, we observed that CTCs in patients with BC were effectively captured and indicated with Pan-CK$^+$ DAPI$^+$ and CD45$^-$ (Figure 3B). With such a high detection rate (86.86%) in malignant BC, the CytoBot® 2000 automated CTCs detection and isolation system seem promising for clinical diagnosis of BC.

Further, we assessed the clinical diagnostic value of CTC in BC progression with clinicopathologic features, including tumour stage, tumour volume, node burden, metastasis, human epidermal growth factor receptor 2 (HER-2) state, and histological grade (Table 1). Among patients with BC, 137 patients were identified with different stages according to the TNM staging system: 1, 29, 57, 43, 7 patients with BC were at 0, I, II, III, and IV stage, respectively (Table 1). We observed that increased CTCs and detection rate were significantly associated with advanced cancer stage ($P = 0.0216$), and the average CTCs count of TNM stage I to IV (1 patient with stage 0 was excluded) were 2.59, 2.81, 4.05, and 4.71, respectively (Table 1). Moreover, tumour size ($P = 0.0349$) and node stage ($P = 0.0103$) were correlated with CTCs count; the CTC positive rate was higher in the advanced tumour stage (Table 1). In addition, more CTCs in HER-2 positive (51 of 137) patients with BC were observed, representing a poorer prognosis [29] than HER-2 negative cases ($P = 0.0201$) (Table 1). The data revealed no correlation between CTC and distal metastasis, although M1 patients had more CTCs than M0 (4.71 > 3.15, Table 1). From these data, we could propose that CTCs were significantly associated with BC progression.

The sensitivity and specificity of CytoBot® 2000 were further assessed through the ROC curve using SPSS 24, and the cut-off value was determined by the Youden index. As shown in Figure 3C and Supplement Table 2-4, the best Youden index are 0.806, 0.236, and 0.561 in ROC of healthy and non-healthy, healthy and benign, and benign and malignant respectively, which indicated the cut-off value of 0.5, 0.5, and 1.5, the sensitivity and specificity of 80.6% and 100%, 63.3% and 100%, and 74.5% and 81.6%, respectively, and area under the curve (AUC) of 0.903, 0.816, and 0.803 respectively. Therefore, the clinical correlation between CTCs and clinical features was further analysed when the cut-off was set to 2 by chi-square analysis in comparison between benign and malignant groups. When cut-off value was 2, the CTCs results revealed a correlation with tumour malignancy ($P < 0.0001$), TNM stage ($P = 0.032$), and tumour size ($P = 0.0326$) but not with node stage, tumour metastasis, HER-2 state, and histological grade (Table 2, Figure 3D). A higher CTC positive rate was presented in patients with an advanced cancer stage (Table 2). Collectively, these data indicated that the CTCs, detected using CytoBot® 2000, exhibited a vital significance for cancer diagnosis, and the platform exhibited an objective performance.

Table 2
Analysis of the clinical relevance of CTCs when the cutoff value is 2.

Subjects	N	CTC≥2	CTC<2	c^2	*p* value
Malignant	137	102	35	47.18	**<0.0001**
Benign	49	9	40		
Cancer stage					
0	1	0	1		
	29	22	7	8.804	**0.032**
	57	36	21		
	46	38	5		
	7	6	1		
Tumor size					
T1	40	28	12	8.766	**0.0326**
T2	71	51	20		
T3	16	15	1		
T4	9	8	1		
Node stage					
N0	62	43	19	5.584	0.1337
N1	31	21	10		
N2	28	23	5		
N3	16	15	1		
Metastasis					
M0	130	96	34	0.4919	0.4831
M1	7	6	1		
HER-2 state					
Positive	51	40	11	0.7626	0.3825
Negative	81	58	23		
Histology grade					
1	6	4	2	0.7222	0.6969

Subjects	N	CTC≥2	CTC<2	c^2	*p* value
2	50	40	10		
3	48	36	12		

3.3 Dynamic change in CTCs in the clinical treatment of BC

Several studies have demonstrated the monitoring value of CTCs in clinical treatments [30]. In the current study, a follow-up study was reviewed in 23 patients with BC. The CTCs were detected pre- (red column) and post-treatment (two times medical observation at least and no more than four times). We observed that 15 of 23 patients (65.22%) presented decreased CTCs in blood, but 1 of 23 patients' CTCs (4.35%) increased with treatment (patient 1). Additionally, 3 of 23 patients' CTCs (13.04%) decreased at first but re-raised in a later period (patient 13, 18, 24), and 4 patients' CTCs (17.39%) did not change dramatically (patient 2, 5, 6, 17) (Figure 4A). In combination with the clinical treatment and medical observations of BC patients in follow-up (details in Supplement excel), cancer development and changes of CTC number were consistent during treatment in 10 of the 15 (73.33%) BC patients with declining CTC levels. MRI is invaluable in medical observation of the follow-up in cancer patients and we also checked the only three sets of MRI images of BC patents in follow-up. This correlated well with clinical examination of reduced tumour MRI obtained from patients 15 and 19 (Figure 4B). Moreover, in 3 patients which the CTCs re-raised in clinical treatment, the tumour recurrence happened in patient 18 after local surgical resection and confirmed by MRI test (Figure 4B).

Combining clinical therapeutic strategies, we observed that 42.86% (3/4), 66.67% (2/3), 60% (3/5), and 83.33% (5/6) patients benefited from chemotherapy (Taxane and Carboplatin), target therapy (Trastuzumab and Pertuzumab), chemotherapy plus target therapy, and other therapies, including hormonotherapy (Anastrozole) and radiotherapy, respectively (Figure 5). The results revealed the poor outcomes of single intervention strategies, especially chemotherapy administration only (Figure 5A). We noted that 2 patients' CTCs were elevated (patient 15 and 13) when radiotherapy intervention followed chemotherapy and target therapy (Figures 5A and B), respectively. However, in patient 3 (Figure 5D), late radiotherapy intervention did not result in CTCs recurrence. Collectively, these results presented the potency of CTCs in clinical surveillance.

3.4 Increasing proportion of $CK^+CD45^+DAPI^+$ CTC during treatment

The prevalence of $CK^+CD45^+DAPI^+$ CTC (dual-positive CTC) occurred in the CTC detections discovered and ignored by most previous studies [31, 32]. However, in recent years, several studies have proposed the clinical significance of dual-positive CTCs [33, 34]. We successfully discovered $CD45^+$ CTCs using CytoBot® 2000 (Figure 6A). Overall, dual-positive CTCs were detected in 43 of 137 patients (31.39%, average 5.20) with malignancy, and 8 of 49 (16.33%, average 3.12) patients with carcinoid ($P = 0.1977$) (Figure 6B). We could not discover the relevance of clinicopathologic features with dual-positive CTCs

(data not shown). Further, we analysed the dynamic change of dual-positive CTCs in the follow-up review. As shown in Figure 6C, 11 of 23 BC patients (47.83%) presented decreasing dynamics of dual-positive CTCs, whereas increased in 6 patients (26.09%, patient 2, 5, 13, 15, 16, 17), and there is no evident change in 5 patients (21.74%, patient 1, 4, 6, 18, 23). A comparison of dynamic changes between CTCs and dual-positive CTCs was performed and showed that 12 patients exhibited similar changes of CTCs and dual-positive CTCs during clinical treatments, including decreasing tendency (10/15, 66.67%, patient 3, 8, 10, 11, 12, 14, 19, 20, 21, 22), fluctuant change (1/3, 33.33%, patient 13) and no change (patient 6). However, 3 cases (patient 16, 15, and 9) presented an opposite tendency in changes CTCs and dual-positive CTCs.

Interestingly, we found that the ratios of dual-positive CTCs to total CTCs in pre-treatment, post-treatment, and 1-month post-treatment were 36.13%, 49.00%, and 65.82%, respectively. We further analysed whether the raised dual-positive CTCs proportion was relevant with clinical treatment strategies. The results shown, the events that increased rate of dual-positive CTCs happened in 57.14% (4/7), 66.67% (2/3), 80% (4/5), and 50% (3/6) of patients which had received chemotherapy, target therapy, chemotherapy plus target therapy, and other therapy, respectively (Figures 6D–G). In general, these data revealed that although the proportion of dual-positive CTCs was elevated after chemotherapy and targeted therapies, but the dual-positive CTCs decreased during clinical treatment indeed.

4 Discussion

The high sensitivity of CTCs detection by CYTOBOT ® 2000

CTCs are rare in the healthy human body, but their amount increases in cancer [35]. In the current study, we observed a 63.27% CTC detection rate even in the benign group using CytoBot® 2000, whereas it increased to 86.86% in the malignant group. In the malignant population, the CTC detection rates were within the range of 82–100%, and a distinct tendency of CTCs conformed to serial indicators of tumour progression, such as tumour size and node burden (Table 1). To assess the sensitivity and specificity of the CytoBot® 2000 CTCs system, statistical analysis based on ROC curves revealed that at a cut-off value of 2, CytoBot® 2000 had 74.5% sensitivity and 81.6% specificity for CTCs detection, with an AUC of 0.807 (Fig. 3C).

Currently, CTCs detection efficiency differs with different technologies [20]. Among the CTCs isolation systems, CellSearch® was the earliest and first FDA-approved platform for CTCs detection. However, the detection efficiency was low, as shown by previous studies. Schindlbeck [21] et al. used CellSearch® to analyse 202 patients in various stages of BC and observed a CTCs detection rate of only 20%. For metastatic BC (MBC), Jacqueline used CellSearch® and detected no more than 50% of CTCs in 112 patients [36]. In addition, Chen et al. examined 366 patients with BC using CytoSorter® (Watson, Hangzhou) and detected CTCs in 15.95% of patients with benign tumours and 85.16% of patients with malignant tumours [37], but CytoSorter® is not capable of distinguishing healthy person from a patient with a benign tumour. Flow cytometric analysis can be applied to CTCs isolation, and Ma et al. identified 80 patients using flow cytometry in 186 patients with BC with a detection rate of 42.78% [38]. There are

increasing platforms for CTC detection with excellent performance that based on microfluidics technology. For example, CelSee and Parsortix are efficient platforms for CTC isolation that are both depend on the cell size and deformability of blood cell. There is difference that the Clearbridge, another device for CTC detection, developed as a spiral microfluidic device and isolated CTC rely on inertia of cells. For ctDNA detection in CTCs, Daskalaki observed that 91 of 165 patients with BC (55.4%) were positive for CK-19 mRNA [39]. To reduce the interference of background cells, Molloy tested 733 patients with BC with CTCs using multiple marker genes; however, the detection rate was only 7.4% [40]. Similar with CelSee and Parsortix, there is the same method basis that the CytoBot 2000 operated in CTC detection. It maybe the best way that the cell size-based filter which used for deletion of background cell. The CytoBot® 2000-assisted detection had a much higher CTC detection rate in benign and malignant groups and is expected to be an effective clinical adjunctive diagnostic platform for BC.

The clinical significance of CTCs

In the current study, we observed that the CTC count in patients with malignant BC was significantly higher than that in the benign group ($P < 0.0001$), and the patients with advanced tumour stage and larger tumour size often carried more CTCs in the circulation system (Table 1). Consistent with our results, in a previous pooled study, CTCs were examined in 3137 non-metastatic patients with BC (stages I–III) using the CellSearch® system and were significantly correlated with TNM stage and tumour size [41]. In another study, Jin [37] analysed 366 patients with BC using the CytoSorter® CTCs isolation system and revealed that CTCs were significantly associated with cancer progression, tumour size, and metastasis except for node stage.

Treatment monitoring based on clinical CTCs dynamics

In this follow-up review, 10 of 15 patients (73.33%) revealed decreased CTCs enumeration and improved tumour progression combined with clinical diagnosis. We observed that the local resection of tumour tissue was the direct cause of CTC reduction, whereas subsequent chemotherapy (carboplatin, paclitaxel, docetaxel), targeted therapy (HER-2 positive only, pertuzumab plus trastuzumab), or radiotherapy could consistently suppress tumour recurrence and CTC migration. Anna-Maria et al. revealed that dynamic changes in CTCs correlated well with the prognosis of 156 patients with BC in a long-term tracking study [42]. Similarly, Yu et al. [43] monitored CTC dynamics in 11 patients with BC and exhibited a significant correlation with clinical progression. We observed that the combination of chemotherapy and target therapy was more efficient than monotherapy. However, the HER-2 target treatment was not suitable for HER-2 negative patients with BC. Therefore, after the end of monotherapy, appropriate radiotherapy or other anti-cancer solutions are more helpful for cancer elimination.

Dual-positive CTCs

Dual-positive CTCs, defined as $CK^{+}CD45^{+}DAPI^{+}$, are mysterious and significant in tumour metastasis, but these are excluded in previous CTCs detections [31] [44]. However, a growing number of investigators believe that dual-positive CTCs may have a more crucial role in cancer development [33, 34, 45]. The

existence of such dual-positive CTCs has been proposed by several hypotheses, in which cancer cells hybridise with another type of cell, mostly macrophages [45]. Charles et al. [46] recently revealed that in vitro colon cancer or glioma cells co-cultured with macrophages could spontaneously initiate cell fusion. Further verification by immunofluorescence, chromosome analysis, and gene expression revealed that cancer cell–macrophage fusion did occur. Injection of the fused cells into mice revealed that the 'hybrid cells' exhibited a greater capacity for cancer migration and tumorigenicity than the parental cancer cells. Daniel and Mu observed that fusing cells had a better prognostic value than conventional CTCs [47], [48]. In our present study, dual-positive CTCs were statistically analysed with clinical examination. Unfortunately, no clear correlation with tumour progression was observed. However, similar dynamics were observed in the follow-up review in CTCs and dual-positive CTCs, and the proportion of dual-positive CTCs increased with clinical treatment.

Comparing the different treatment schemes, we observed that although the combination of chemotherapy and targeted therapy was more effective, it also resulted in an increased proportion of dual-positive CTCs. At present, $CD45^{+}$ CTCs have been proved that especial for chemoresistance [49, 50]. Yang et al. [51] illustrated that the doxorubicin resistance ability dramatically increased in fusion cells than in only MCF7 cells. Nagler et al. [52] discovered that the fusion of cancer cells with bone marrow-derived cells contributes to the expression of drug resistance genes, *ABCB1a* and *ABCB1b*. Hence, the high dual-positive CTCs proportion in clinical treatment results from chemoresistance. Further sequencing is essential for uncovering this dynamic change.

However, this study has some limitations. CTCs consist of multiple types, including epithelial, mesenchymal, and cell clusters, and mesenchymal CTCs are more pro-metastatic [53, 54]. The CytoBot® 2000 system used in the current study were specific for epithelial CTCs isolation (EpCAM) but not mesenchymal CTCs. Satelli et al. [55] used an epithelial (EpCAM) and mesenchymal (vimentin) capture system for CTCs in metastatic BC and revealed that there were significantly more mesenchymal CTCs than epithelial CTCs in patients with advanced stage. The CytoBot® 2000 also supports the detection of mesenchymal CTCs. In further studies, both phenotypes of CTCs must be studied better to understand the relationship between CTCs and clinical features. Additionally, further study with next-generation sequencing is essential to clarify the mechanism of dual-positive CTCs dynamics, including target treatment and neoadjuvant therapy, respectively.

5 Conclusions

In the present retrospective study, a novel CTCs isolation and enrichment system, CytoBot® 2000, was used to detect CTCs in 186 patients with BC at different stages. Data analysis was performed in association with clinicopathological features. We observed that CTCs could effectively differentiate the benign population from a malignant one. In addition, 23 patients with BC were enrolled in a follow-up study, and most of the patient's CTCs dynamics were valuable for clinical monitoring. The combined administration of chemotherapy and targeted therapy inhibited tumour progression efficiently, but increased the proportion of dual-positive CTCs has been found in this work with small sample size that

need to be verified further. In summary, we demonstrated that CTCs have an indispensable role and significance as clinical diagnostic indicators for BC, and the CytoBot® 2000, as an advanced automated CTCs isolation and enrichment platform, holds promise as a powerful aid for clinical application.

Declarations

Ethics approval and consent to participate

The current study has followed the Declaration of Helsinki, and the research was approved by the Ethics Committee of Fudan University Shanghai Cancer Center (050432-4-1911D) and The First Affiliated Hospital of Soochow University (2021050). All the patients agreed and signed the informed consent forms.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article [and supplementary information]

Conflict of Interest

Author Jie Wang and Jing Yan were employed by the company Holosensor Medical Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Gong Zhiyun, Yan Jing, Chen Yan: Roles/Writing - original draft experiment performance, Data analysis. Lu Renquan, Yan Jing, Chen Yan: Funding acquisition, Project administration Yan Jing, Wang Jie: Methodology, Investigation. Li Aoshuang, Yang Xiaorong, Shi Weizhong: Sample enrollment, Statistics. Guo Lin, Dai Tiantian: Validation.

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Figures

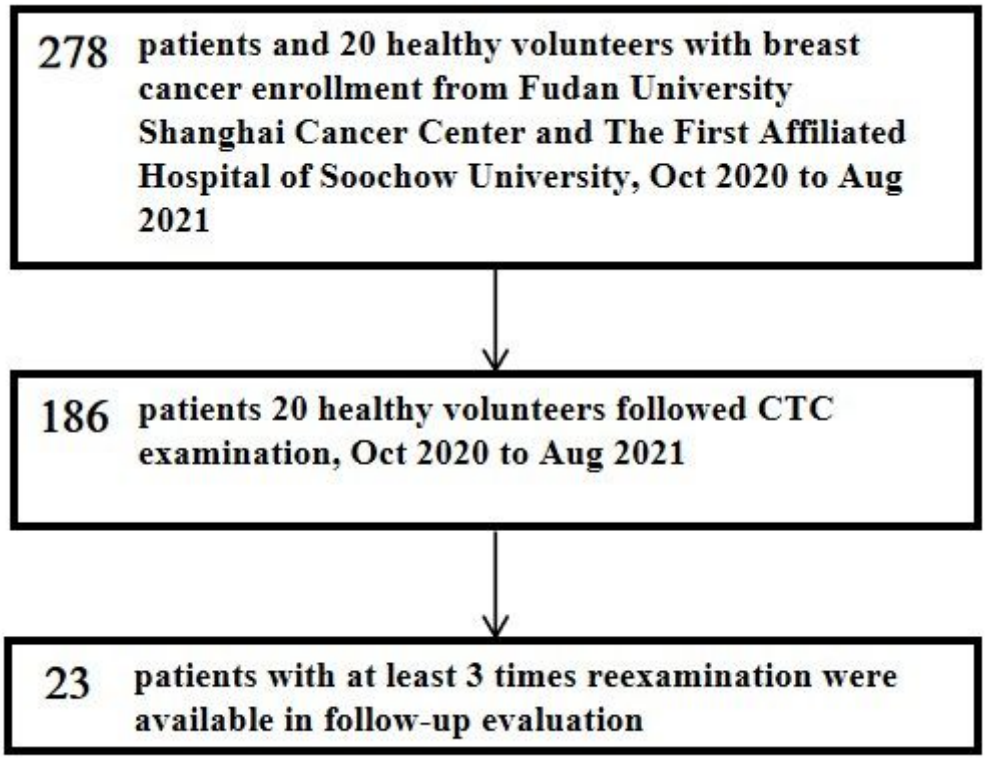

Figure 1

Flow chart of the study. In this retrospective study, 278 patients with BC from University Shanghai Cancer Center and The First Affiliated Hospital of Soochow University were tested for CTCs detection using CytoBot® 2000 from October 2020 to August 2021. Overall, 92 patients were excluded because of the unavailability of baseline information. In total, 23 patients were available for CTCs dynamic analysis.

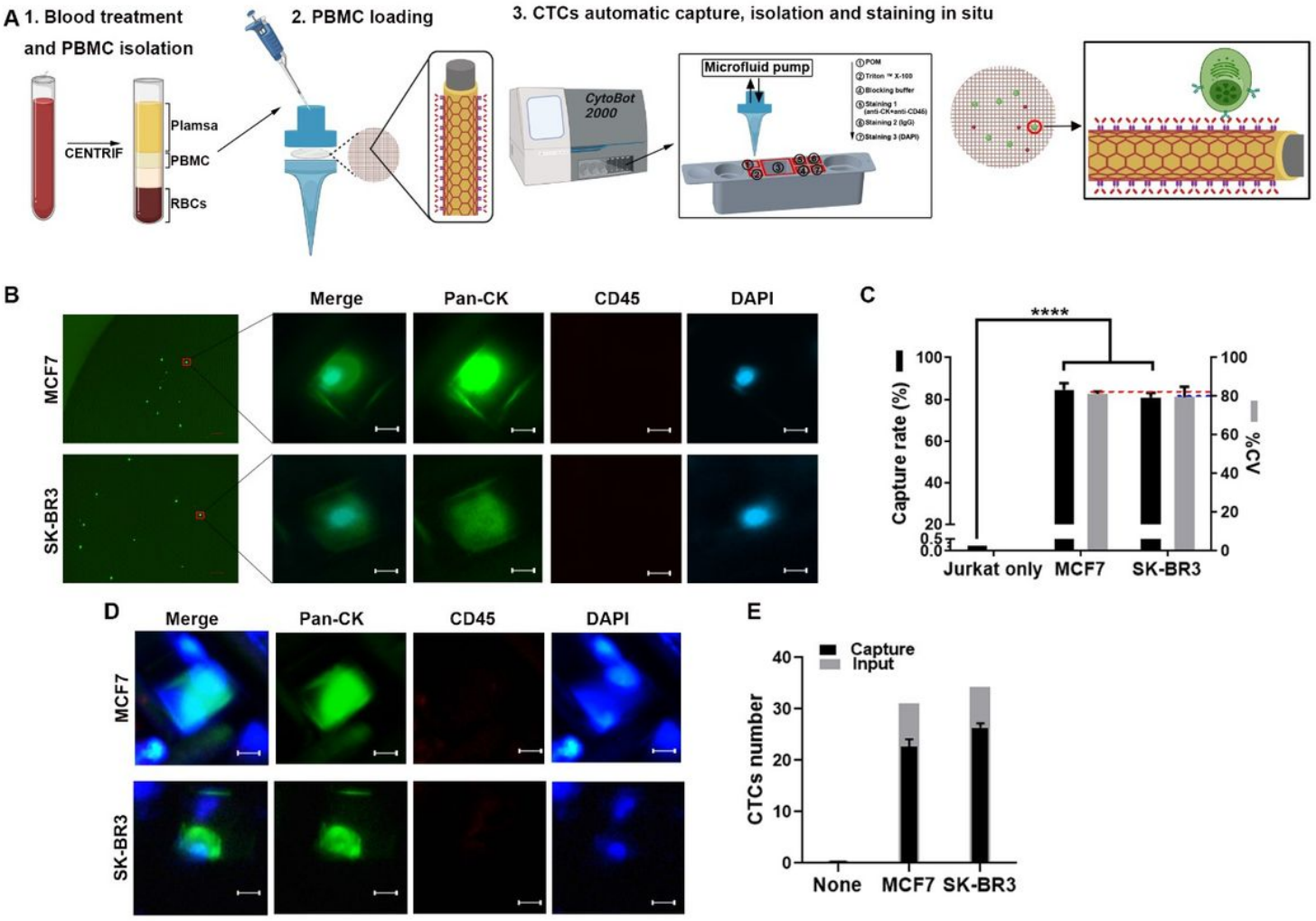

Figure 2

BC cells detection and identification with CytoBot® 2000. (A) The procedure of CTCs detection and capture mechanism in CytoBot® 2000. CytoBot® 2000 is an automatic CTCs capture and isolation system which uses PBMC obtained from peripheral blood of patients with BC. CTCs were captured with customised chip designed according to CTC's features, such as diameter and surface marker. (B) BC cells, MCF7, and SK-BR-3, captured and identified with CytoBot® 2000, shown as Pan-CK$^+$ (green), DAPI$^+$ (blue), and CD45$^-$ (red) in immunofluorescence images, respectively. Scale bars are 50 and 5 μm, respectively. (C) The capture rates and precision of CytoBot® 2000. MCF7 and -SK-BR-3 cells (50–60) were mixed in background cells (Jurkat cell, 1 million) and suspended in 300 μL PBS. Cancer cells were isolated. Precision is shown as %CV. (D and E) Spike assay was performed for performance validation of this device, 30-40 of cancer cells were added in blood and then followed CTC enrichment. Scale bars = 5 μm. One-way ANOVA, n = 3. The results are presented as mean ± S.E.M., $^{**}P < 0.01$.

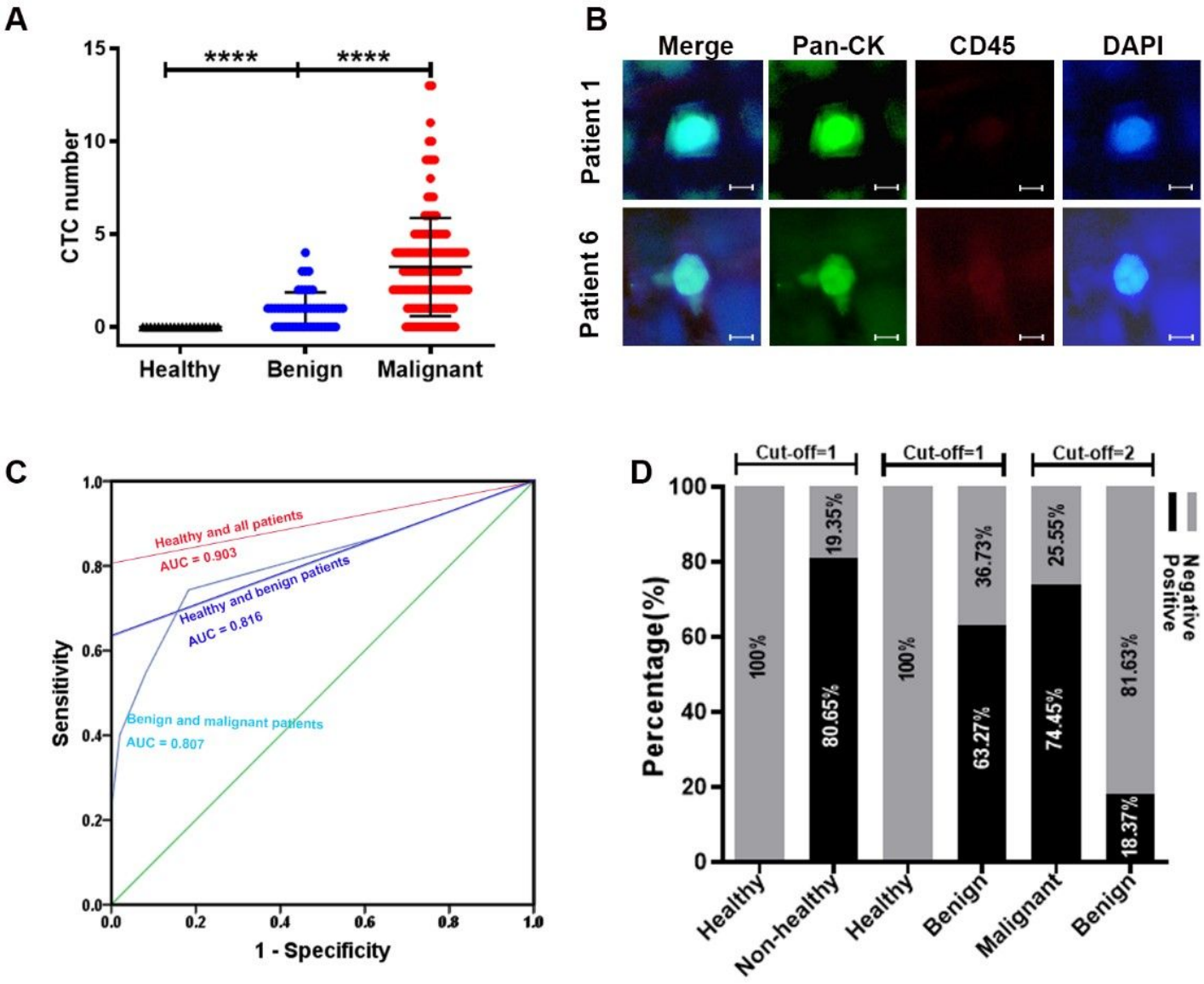

Figure 3

CTCs detection in patients with BC. (A) CTCs were detected in healthy normal (n=20), benign (n = 49) and malignant (n = 137) groups; unpaired t test, ****$P < 0.0001$. (B) CTCs were indicated by immunofluorescence images isolated form patient 1 and 6 with BC, scale bar: 5 μm. (C) ROC curve of CytoBot® 2000 was generated using CTCs count between healthy and non-healthy groups, healthy and benign groups, malignant and benign groups. (D) CTCs positive percentage in healthy and non-healthy groups, healthy and benign groups, malignant and benign patients when cut-off value was 1, 1, and 2 respectively.

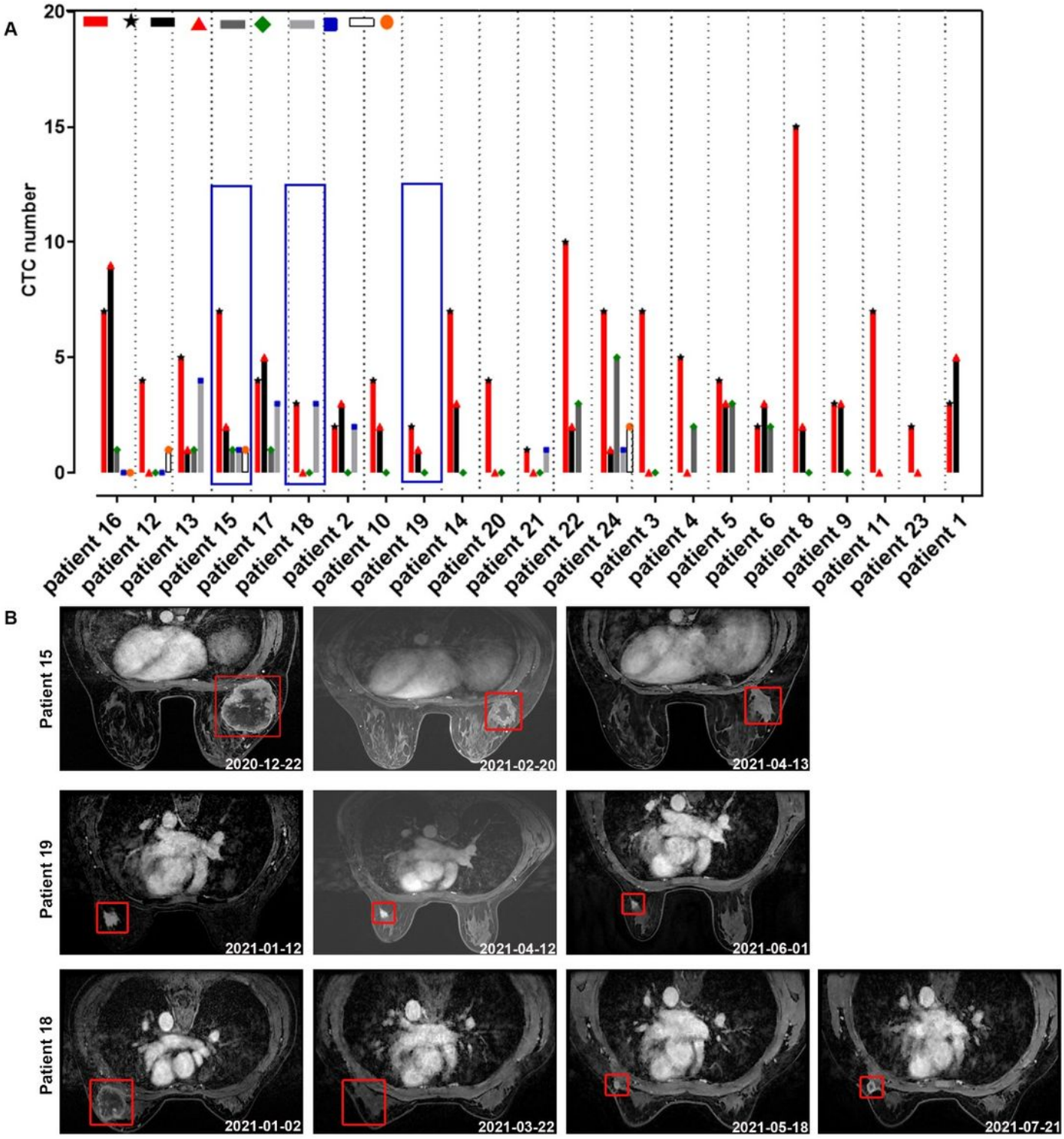

Figure 4

Dynamic changes in CTCs in clinical treatment of BC. (A) The change of CTC number in 23 patients of follow-up. The red bar means the first diagnosis in hospital; the black, the grey, the light grey, and the white bars means the four times medical observation after therapy, respectively. (B) Magnetic resonance imaging of patients 15, 18, and 19.

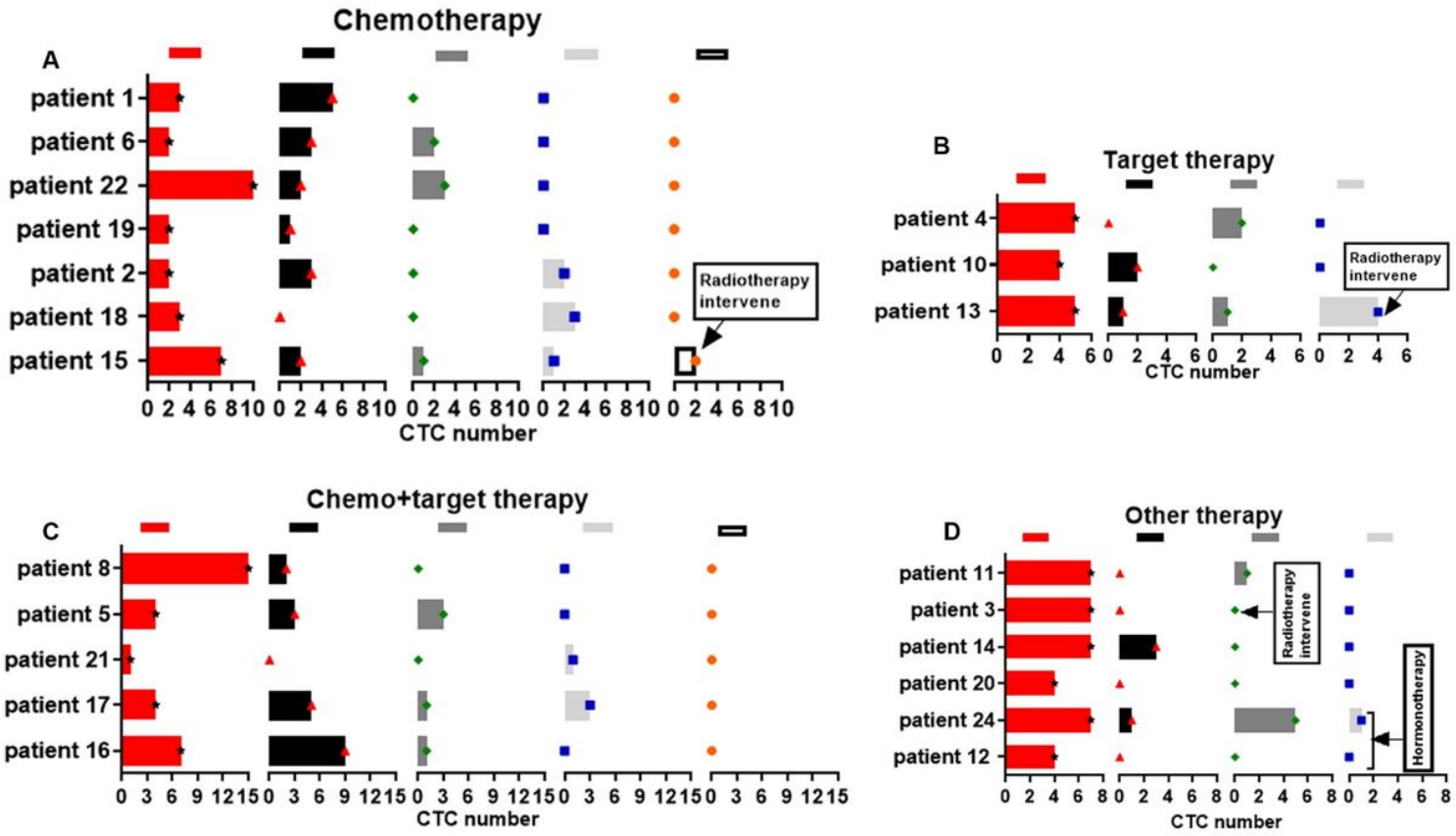

Figure 5

Changes in CTCs as per different clinical therapeutic strategies. CTCs dynamics of 21 patients with BC were divided into 4 groups depending on different treatment strategies: (A) chemotherapy (n = 7), (B) target therapy (n = 3), (C) chemotherapy combined with target therapy (n = 5), and (D) other therapy, including hormonotherapy and radiotherapy (n = 6). The change of CTC number was indicated by the bar that colored with red, black, grey, light grey, and white that means the first diagnosis in hospital and four times medical observation after therapy, respectively.

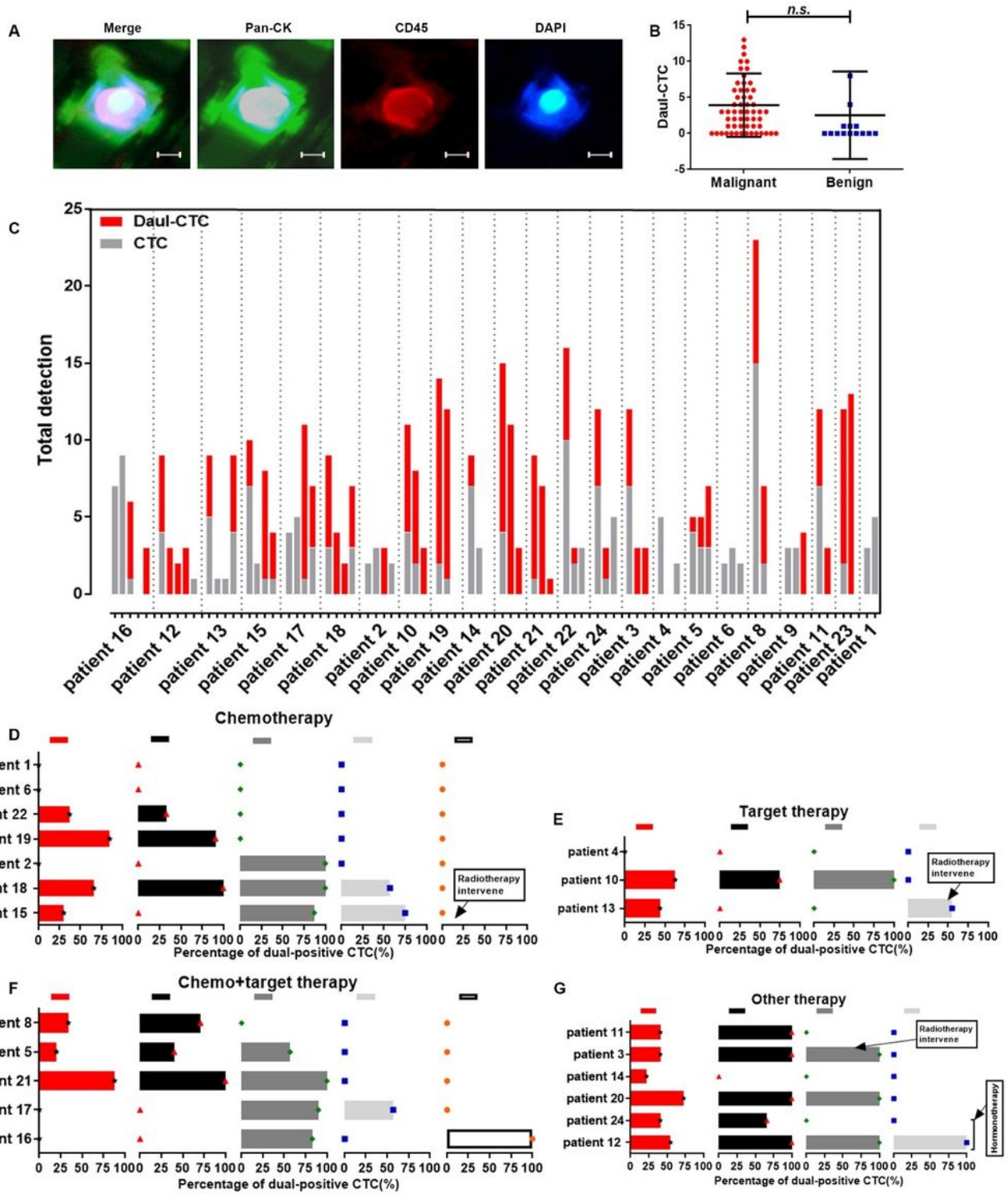

Figure 6

Increased rate of $CK^+CD45^+DAPI^+$ CTCs during treatment. (A) Dual-positive CTCs in immunofluorescence images; Pan-CK^+ (green), $CD45^+$ (red), and $DAPI^+$ (blue), scale bar: 5 μm. (B) Dual-positive CTCs detection in malignant (n = 137) and benign (n = 49) group; unpaired t test was performed, n.s., no significant difference. (C) Combination of CTCs and dual-positive CTCs in follow-up review. Red bar indicates dual-positive CTCs, and grey bar indicates CTCs. The CTC number of four times medical observation in a

single patient were shown with four consecutive diagrams (left-to-right, between dotted lines). Dynamic percentage of dual-positive CTCs from 21 patients with BC grouped into (D) chemotherapy (n = 7), (E) target therapy (n = 3), (F) chemotherapy combined with target therapy (n = 5), (G) other therapy, including hormonotherapy and radiotherapy (n = 6). The proportion change of dual-positive CTC number in first diagnosis and four times medical observation was indicated by the bar that colored with red, black, grey, light grey, and white, respectively.

Supplementary Files

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- SupplementexcelFollowup.xlsx
- Supplementtables.docx