Am J Transl Res 2023;15(3):1941-1952
www.ajtr.org /ISSN:1943-8141/AJTR0148755

Original Article

Correlation between the expression of CD24 on circulating tumor cells and prognosis in breast cancer

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Received December 26, 2022; Accepted February 11, 2023; Epub March 15, 2023; Published March 30, 2023

Abstract: Objective: To investigate the expression of tumor stem cell marker CD24 in peripheral blood circulating tumor cells (CTCs) of breast cancer and the value of CTCs in predicting the prognosis of breast cancer patients. Methods: Clinical data of 102 breast cancer patients from January 2015 to December 2019 were retrospectively collected. CTC test results, CD24 test results, tumor size, tumor stage, pathological type, molecular type, lymph node metastasis, survival time, and survival status of patients were collected. The correlation between the expression of CD24 in peripheral blood CTCs of breast cancer and the survival time of patients was analyzed. Results: Epithelial-CTCs were closely related to estrogen receptor (ER) expression (P = 0.036) and TNM stage (P = 0.018). Mixed epithelial/mesenchymal-CTCs were closely related to lymph node metastasis in breast cancer patients (P = 0.026). There was no obvious correlation between mesenchymal-CTCs and clinical characteristics (P > 0.05). The positive expression rate of CD24 in CTCs was 58.82% (60/102). The number of CD24-positive CTCs was closely related to TNM stage (P = 0.002), lymph node metastasis (P = 0.020), and tumor size (P = 0.025). The cumulative survival rate of patients with CD24-positive CTCs > 1.5/5 ml (73%) was significantly worse than that of patients with CD24-positive CTCs ≤ 1.5/5 ml (88%) (P < 0.05). There was no significant difference in the cumulative survival rate between patients with mixed-CTCs > 2.5/5 ml (72%) and patients with mixed-CTCs ≤ 2.5/5 ml (87%) (P = 0.336). The cumulative survival rate of patients with CD24-positive mixed-CTCs > 0.5/5 ml (72%) was significantly lower than that of patients with CD24-positive mixed-CTCs ≤ 0.5/5 ml (92%) (P < 0.05). Conclusion: The positive expression of CD24 in CTC is closely related to TNM stage, lymph node metastasis, and tumor size in breast cancer patients. The positive expression of CD24 in CTCs, especially in mixed-CTCs, may be one of the prognostic indicators for patients with early and intermediate stage breast cancer.

Keywords: Circulating tumor cells, epithelial-mesenchymal transition, CD24, breast cancer, prognosis

Introduction

Breast cancer is one of the most common malignancies in women [1], and despite significant advances in treatment regimens, screening, and surveillance, the risk of disease recurrence persists for years after diagnosis [2]. Local and systemic breast cancer recurrence can be difficult to detect early due to the lack of relevant clinical symptoms and limitations of radiologic detection. Therefore, there is considerable interest in novel noninvasive methods for predicting breast cancer prognosis.

Liquid biopsy is a non-invasive technique that can yield important diagnostic, therapeutic, and prognostic information in many types of cancer. A component of liquid biopsies called circulating tumor cells (CTCs) has shown promise in detection and diagnosis of breast cancer [3]. CTCs are shed from the primary tumor site and enter the circulation through the blood or lymphatic system. Epithelial-mesenchymal transition (EMT) can occur when tumor cells enter the peripheral blood circulation. Numerous studies have shown that epithelial-mesenchymal transition (EMT) is a key biologic process for tumor cells to acquire the ability to invade and metastasize [4]. The expression of CTC epithelial markers wis down-regulated or even disappears during EMT. According to the expression of EMT markers, CTCs can be divided into epithelial type (epithelial-CTCs), mesenchymal type (mesenchymal-CTCs) and mixed epithelial/mesenchymal type (mixed-CTCs). Some scholars have carried out EMT typing of

CTCs enriched in peripheral blood of breast cancer patients by multiplex RNA in situ hybridization, and found that interstitial CTCs are more related to tumor progression and treatment tolerance [5].

CD24 is a highly glycosylated glycosylphosphatidylinositol-anchored surface protein that is overexpressed in various cancers [6-8]. In breast cancer, the expression of CD24 was significantly higher in invasive cancer than in normal tissues [9]. Expression of CD24 on the cell surface and in the cytoplasm was associated with tumor size, histologic grade, lymph node positivity, and poor prognosis [10]. However, no study has yet evaluated the correlation between the expression of CD24 in CTCs and the prognosis of breast cancer. In terms of CTC detection, CanPatrol's second-generation CTC enrichment technology breaks through conventional detection methods, has high sensitivity for CTC detection and EMT typing and identification, and can identify and detect CTCs of epithelial type, mesenchymal type, and mixed type. CanPatrol has the characteristics of high detection rate and comprehensive detection of all types of CTCs. Based on this, in order to further explore the influence of CD24 expression on CTCs for early and middle stage breast cancer prognosis, we used CanPatrol CTC typing detection technology We analyzed the expression of CD24 in different subtypes of CTCs and its correlation with prognosis.

Materials and methods

Patient samples

The clinical data of 102 breast cancer patients who underwent peripheral blood circulating tumor cytology test in the Department of General Surgery, the Second Xiangya Hospital of Central South University from January 2015 to December 2019 were retrospectively collected. All of them were female, and their age was 48.41±10.09 years old (27-75 years old). Inclusion criteria: (1) Patients with pathologically confirmed breast cancer. (2) Patients with tumor node metastasis (TNM) Stage 0-Stage III. (3) Patients between 20 and 80 years old. (4) Patients with no contraindications to chemotherapy. (5) Patients with no history of malignant tumors in other organs. Exclusion criteria: (1) Patients with inflammatory breast cancer or breastfeeding breast cancer. (2) Patients with incomplete clinical data. (3) Patients in whom the mass had been removed prior to admission.

Follow-up of patients began after diagnosis. The content of follow-up included the general condition of the patient, palpation of the affected chest wall (affected breast in breast-conserving patients), palpation of the contralateral breast, mammography of the breast, ultrasonography of bilateral supraclavicular fossa and axillary fossa, CT examination of the lungs, B ultrasound examination of liver and gallbladder, ultrasonography of uterus and adnexa, blood routine, hepatic and renal function examination, and the review of CTC detection. Follow-up time: After diagnosis, the patients were followed up every 3 months until December 2021. Follow-up methods: Patients were regularly revisited and followed up according to the data during each outpatient or hospitalization period. Patients who could not be followed up face to face were followed up by telephone. The overall follow-up rate was 96.0%. The primary outcome was cumulative survival rate. Cumulative survival rate = (number of patients who were alive after N months of follow-up/number of patients who started follow-up) × 100%.

Specimen collection

After the diagnosis of breast cancer by pathologic biopsy, peripheral venous blood was collected to detect CTC, and immunohistochemistry was performed to detect the expression level of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor (HER-2), and proliferating cell associated nuclear antigen (Ki67). The relationship between CD24 expression in preoperative circulating tumor cells of breast cancer patients and patient age, tumor size, tumor stage, pathologic type, molecular type, lymph node metastasis, and the status of ER, PR, Ki67, and HER-2 was analyzed. This study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University (Approval No. 2022-073-02).

CTC and CD24 detection

The optimized CanPatrol CTC enrichment technique was used in this study. (1) Separation of CTCs by cell morphology: 5 ml of venous blood

was drawn from patients on an empty stomach. Erythrocyte lysate (154 mM ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KH\text{-}CO_3$), and 0.1 mM ethylenediamine tetraacetic Acid (EDTA) in deionized water) was used to lyse erythrocytes. The remaining cells were then resuspended in 4% formaldehyde in PBS for 5 min. The cell suspension was transferred to a filter tube, and the cells were filtered by a vacuum pump with a fixed pressure. (2) Three-color RNA in situ hybridization detection: The detection was carried out on a 24-well plate, and the cells on the membrane surface were treated with protease, and then capture probes (respectively for epithelial biomarkers EpCAM, CK8/18/19; mesenchymal cells) were used. Biomarkers (vimentin, twist) were hybridized for 2 h at 42°C. Unbound probes were washed three times with 1000 μl of wash buffer. Then, 100 μl of pre-amplification solution was added to the cells and incubated at 42°C for 20 min. The membrane was cooled, washed 3 times with 1000 μl of wash buffer, and incubated again with 100 μl of amplification solution. Three types of fluorescently labeled probes, Alexa Fluor 594, Alexa Fluor 488, and Alexa Fluor 647, were added and incubated again at 42°C for 20 min. After washing again, the remaining cells were stained with DAPI for 5 min. The expression of EMT markers in CTC was observed by fluorescence microscope (red fluorescence represents epithelial markers, green fluorescence represents mesenchymal markers, and white fluorescence represents CD45 markers). CTC of each patient was identified and classified according to the identification of markers, and CTCs ≥ 1 was defined as positive in this study.

The expression of CD24 in CTC was detected by the same steps as above, and CD24 mRNA probe was used on the basis of CTC enrichment, which was labeled with fluorescent labeled probe and marked purple. The criteria for the expression of CD24 in CTC of different EMT types were as follows: 1-2 signal points were low expression, 3-9 signal points were moderate expression, and ≥ 10 signal points were high expression.

Treatment options for breast cancer patients

All patients underwent surgery. Postoperative adjuvant therapy mainly included the following schemes: There were 9 cases of Doxubicin+Cyclophosphamide (AC) scheme, 22 cases of Doxubicin+Cyclophosphamide→Docetaxel (AC-T) scheme, 5 cases of Doxubicin+Cyclophosphamide→Docetaxel+Trastuzumab (AC-TH) scheme, 7 cases of Doxubicin+Cyclophosphamide→Docetaxel+Trastuzumab+Paclitaxel (AC-THP) scheme, 15 cases of dose dense Doxubicin+Cyclophosphamide→Docetaxel (ddAC-T) scheme, 2 cases of dose dense Doxubicin+cyclophosphamide→Docetaxel+Trastuzumab (ddAC-TH) scheme, 8 cases of (Doxubicin+Cyclophosphamide→Paclitaxel) AC-wP scheme, 1 case of Decitabine+Paclitaxel (DAC-wP) scheme, 16 cases of (Docetaxel+Cyclophosphamide) TC scheme, and 5 cases of TAC scheme. There were 1 case of Epirubicin+Cyclophosphamide→Paclitaxel (EC-P) regimen, 1 case of Paclitaxel (wP) scheme, 1 case of Trastuzumab - emtansine conjugate (TDM1) scheme, 1 case of letrozole alone, 2 cases of radiotherapy, and 6 cases of postoperative untreated treatment.

Statistical methods

SPSS 23.0 statistical software was used for statistical processing of data. Enumerated data were described by n (%), and differences between groups were compared by χ^2 test. Measured data were described using mean ± standard deviation ($\bar{x} \pm s$), and t-test was used to compare the differences between groups. Receiver operating characteristic (ROC) curve was used to analyze the predictive power of CD24 expression on the prognosis of breast cancer, and the optimal cut-off value was calculated according to specificity and sensitivity. Survival was compared between different groups using the Kaplan-Meier method. The Cox regression model was used to analyze the regression relationship between survival events and multiple risk factors. $P < 0.05$ was considered significant. The main outcome measure was the relationship between CD24 expression and patients' survival, and the secondary outcome measure was the relationship between different types of CTCs and patients' survival.

Results

Expression of CD24 in different types of CTC

Fluorescence microscopy images of different subtypes of CTCs are shown in **Figure 1**. Epi-

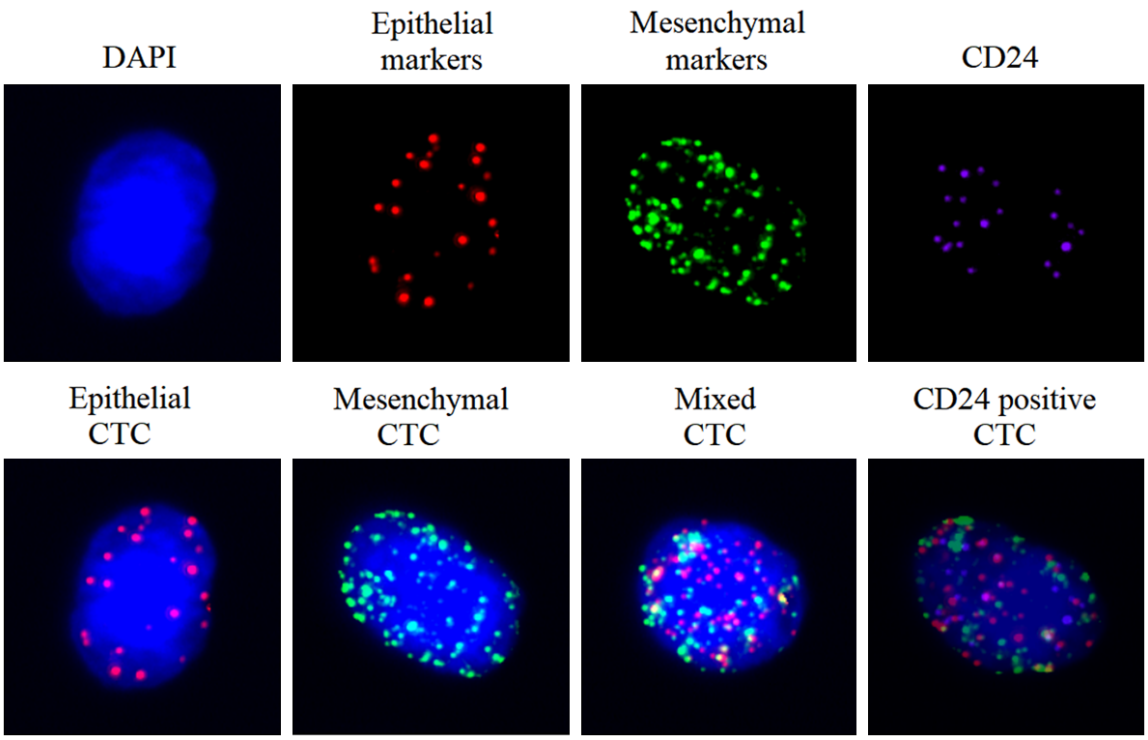

Figure 1. Fluorescence microscopy images of different subtypes of CTCs. Blue fluorescence: DAPI nucleus; Red fluorescence: epithelial marker expression signal point; Green fluorescence: mesenchymal cell marker expression signal point; Purple fluorescence: CD24 gene expression signal point. Circulating Tumor Cells (CTCs).

thelial-CTCs were detected in 64 of 102 patients; the total number of Epithelial-CTCs was 147. Mixed-CTCs were detected in 75 of 102 patients; the total number of Mixed-CTCs was 296. Mesenchymal-CTCs were detected in 46 of 102 patients; the total number of Mesenchymal-CTCs was 94. The expression of CD24 in different subtypes of CTC is shown in **Table 1**.

Correlation of CD24 expression with clinical factors in breast cancer

Epithelial-CTCs were closely related to ER expression (P = 0.036) and TNM stage (P = 0.018), as shown in **Table 2**. There was no significant correlation between mesenchymal-CTCs and clinical characteristics (age, tumor location, tumor size, pathological type, T stage, N stage, molecular type, lymph node metastasis, and expression levels of ER, PR, HER-2, and Ki67), as shown in **Table 3**. Mixed-CTCs were closely related to lymph node metastasis (P = 0.026), but had no significant correlation with age, tumor location, tumor size, pathological type, T stage, N stage, molecular type, ER expression, PR expression, HER-2 expression, or Ki67 expression (all P > 0.05), as shown in **Table 4**. The positive rate of CD24 expression in CTCs was 58.82% (60/102); CD24-positive CTCs were closely related to TNM stage (P = 0.002), lymph node metastasis (P = 0.020) and tumor size (P = 0.025), as shown in **Table 5**.

Correlation of CD24-positive CTCs and mixed-CTCs with lymph node metastasis

The area under the curve (AUC) of CD24-positive CTCs in diagnosing lymph node metastasis was 0.598 (95% CI: 0.484-0.712), P = 0.092, cut-off value = 1.5. The AUC of mixed-CTCs in diagnosing lymph node metastasis was 0.644 (95% CI: 0.535-0.754), P = 0.013, cut-off value = 2.5. The AUC of CD24 positive mixed-CTCs in diagnosing lymph node metastasis was AUC = 0.601 (95% CI: 0.490-0.712), P = 0.084, cut-off value = 0.5, as shown in **Figure 2**.

Correlation between CD24 expression and overall survival in breast cancer patients

Ninety-seven patients completed follow-up, the follow-up rate was 96.0%, and the median follow-up time was 38 months. The groups were

Table 1. Expression of CD24 in different types of CTCs (n %)

Subtype	Total CTCs	CD24 high expression count	CD24 medium expression count	CD24 low expression count	No CD24 expression count	*P* value
E-CTCs	147	6 (4.08%)	18 (12.24%)	41 (27.89%)	82 (55.78%)	0.063
M-CTCs	94	1 (1.06%)	9 (9.57%)	30 (31.91%)	54 (57.45%)	
mixed-CTCs	296	9 (3.04%)	55 (18.58%)	101 (34.12%)	131 (44.26%)	

Note: E-CTCs is Epithelial circulating tumor cells; M-CTCs is mesenchymal circulating tumor cells; mixed-CTCs is epithelial and mesenchymal mixed typic circulating tumor cells.

Table 2. Correlation analysis between Epithelial circulating tumor cells detection and clinical characteristics

Clinical characteristic		Epithelial-CTCs		
		≥ 1 (n = 64)	< 1 (n = 38)	*P*
Age (years)	≥ 50	25 (39.06)	20 (52.63)	0.182
	< 50	39 (60.94)	18 (47.37)	
Tumor site	Left	30 (46.88)	21 (55.26)	0.413
	Right	34 (53.12)	17 (44.74)	
Tumor size	≥ 3 cm	33 (51.56)	17 (44.74)	0.505
	< 3 cm	31 (48.44)	21 (55.26)	
Pathologic type	Carcinoma in situ	1 (1.56)	1 (2.63)	0.865
	Micro infiltration	1 (1.56)	1 (2.63)	
	Infiltration	62 (96.88)	36 (94.74)	
T stage	T1	24 (37.50)	14 (36.84)	0.991
	T2	37 (57.81)	22 (57.90)	
	T3	3 (4.69)	2 (5.26)	
N stage	N0	25 (39.06)	17 (44.74)	0.066
	N1	28 (43.75)	8 (21.05)	
	N2	6 (9.38)	9 (23.68)	
	N3	5 (7.81)	4 (10.53)	
TNM stage	I	9 (14.06)	9 (23.69)	0.018
	II	42 (65.63)	14 (36.84)	
	III	13 (20.31)	15 (39.47)	
Molecular subtyping	Luminal A	6 (9.38)	5 (13.16)	0.272
	Luminal B HER-2 positive	5 (7.81)	7 (18.42)	
	Luminal B Her-2 negative	23 (35.94)	14 (36.84)	
	HER-2 positive	14 (21.87)	8 (21.05)	
	Triple-negative	16 (25.00)	4 (10.53)	
Lymphatic metastasis	with	39 (60.94)	21 (55.26)	0.573
	without	25 (39.06)	17 (44.74)	
ER expression	positive	25 (39.06)	26 (68.42)	0.036
	negative	29 (45.31)	12 (31.58)	
PR expression	positive	31 (48.44)	20 (52.63)	0.682
	negative	33 (51.56)	18 (47.37)	
HER-2 expression	positive	34 (53.12)	25 (65.79)	0.210
	negative	30 (46.88)	13 (34.21)	
Ki67 expression	high	20 (31.25)	9 (23.68)	0.413
	low	44 (68.75)	29 (76.32)	

Table 3. Correlation analysis between mesenchymal circulating tumor cell detection results and clinical characteristics

Clinical characteristic		Mesenchymal-CTCs		
		≥ 1 (n = 46)	< 1 (n = 56)	*P*
Age (years)	≥ 50	21 (45.65)	24 (42.86)	0.777
	< 50	25 (54.35)	32 (57.14)	
Tumor site	Left	21 (45.65)	30 (53.57)	0.426
	Right	25 (54.35)	26 (46.43)	
Tumor size	≥ 3 cm	25 (54.35)	25 (44.64)	0.329
	< 3 cm	21 (45.65)	31 (55.36)	
Pathological type	Carcinoma in situ	1 (2.17)	1 (1.79)	0.430
	Micro infiltration	0 (0)	2 (3.57)	
	Infiltration	45 (97.83)	53 (94.64)	
T stage	T1	16 (34.78)	22 (39.29)	0.742
	T2	27 (58.70)	32 (57.14)	
	T3	3 (6.52)	2 (3.57)	
N stage	N0	17 (36.96)	25 (44.64)	0.174
	N1	19 (41.30)	17 (30.36)	
	N2	4 (8.70)	11 (19.64)	
	N3	6 (13.04)	3 (5.36)	
TNM stage	I	7 (15.22)	11 (19.64)	0.757
	II	27 (58.70)	29 (51.79)	
	III	12 (26.08)	16 (28.57)	
Molecular subtyping	Luminal A	5 (10.87)	6 (10.71)	0.555
	Luminal B HER-2 positive	3 (6.52)	9 (16.07)	
	Luminal B Her-2 negative	16 (34.78)	21 (37.50)	
	HER-2 positive	11 (23.91)	11 (19.64)	
	Triple-negative	11 (23.91)	9 (16.07)	
Lymphatic metastasis	with	29 (63.04)	31 (55.36)	0.433
	without	17 (36.96)	25 (44.64)	
ER expression	positive	25 (54.35)	36 (64.29)	0.308
	negative	21 (45.65)	20 (35.71)	
PR expression	positive	20 (43.48)	31 (55.36)	0.233
	negative	26 (56.52)	25 (44.64)	
HER-2 expression	positive	29 (63.04)	30 (53.57)	0.335
	negative	17 (36.96)	26 (46.43)	
Ki67 expression	high	14 (30.43)	15 (26.79)	0.684
	low	32 (69.57)	41 (73.21)	

Tumor node metastasis (TNM); estrogen receptor (ER); progesterone receptor (PR); human epidermal growth factor receptor (HER-2); circulating tumor cells (CTCs).

merged according to the cut-off value obtained from the ROC curve analysis. The cumulative survival rate of patients with CD24 positive CTCs > 1.5/5 ml (73%) was significantly lower than that of patients with CD24 positive CTCs ≤ 1.5/5 ml (87%) ($P < 0.05$). There was no significant difference in cumulative survival rate between patients with mixed-CTCs > 2.5/5 ml and ≤ 2.5/5 ml (72% vs. 87%, $P = 0.336$). The cumulative survival rate of patients with CD24 positive mixed-CTCs > 0.5/5 ml (72%) was lower than that of patients with mixed-CTCs ≤ 0.5/5 ml (92%) ($P < 0.05$), as shown in **Figure 3**. Multiple variate Cox regression results showed that the hazard ratio (HR) of CD24 positive CTCs was 1.205, the HR of mixed-CTCs was 0.701, and the HR of CD24 positive mixed-CTCs was 10.061, $P > 0.05$, as shown in **Table 6**.

Table 4. Correlation analysis between mixed type circulating tumor cell detection results and clinical characteristics

Clinical characteristic		Mixed-CTCs		
		≥ 1 (n = 75)	< 1 (n = 27)	*P*
Age (years)	≥ 50	35 (46.67)	10 (37.04)	0.388
	< 50	40 (53.33)	17 (62.96)	
Tumor site	Left	35 (46.67)	16 (54.55)	0.262
	Right	40 (53.33)	11 (45.45)	
Tumor size	≥ 3 cm	41 (54.67)	9 (33.33)	0.057
	< 3 cm	34 (45.33)	18 (66.67)	
Pathological type	Carcinoma in situ	1 (1.33)	1 (3.70)	0.553
	Micro infiltration	1 (1.33)	1 (3.70)	
	Infiltration	73 (97.34)	25 (92.60)	
T stage	T1	30 (40.00)	8 (29.63)	0.556
	T2	41 (54.67)	18 (66.67)	
	T3	4 (5.33)	1 (3.70)	
N stage	N0	26 (34.67)	16 (54.55)	0.165
	N1	30 (40.00)	6 (22.22)	
	N2	12 (16.00)	3 (11.11)	
	N3	7 (9.33)	2 (7.41)	
TNM stage	I	14 (18.67)	4 (14.81)	0.617
	II	39 (52.00)	17 (62.96)	
	III	22 (29.33)	6 (22.22)	
Molecular subtyping	Luminal A	7 (9.33)	4 (14.81)	0.838
	Luminal B HER-2 positive	10 (13.33)	2 (7.41)	
	Luminal B Her-2 negative	27 (36.00)	10 (37.04)	
	HER-2 positive	17 (22.67)	5 (18.52)	
	Triple-negative	14 (18.67)	6 (22.22)	
Lymphatic metastasis	with	49 (61.33)	11 (40.74)	0.026
	without	26 (38.67)	16 (59.26)	
ER expression	positive	36 (48.00)	16 (59.26)	0.316
	negative	39 (52.00)	11 (40.74)	
PR expression	positive	37 (49.33)	14 (51.85)	0.822
	negative	38 (50.67)	13 (48.15)	
HER-2 expression	positive	45 (60.00)	14 (51.85)	0.462
	negative	30 (40.00)	13 (48.15)	
Ki67 expression	high	20 (26.67)	9 (33.33)	0.510
	low	55 (73.33)	18 (66.67)	

Tumor node metastasis (TNM); circulating tumor cells (CTCs); estrogen receptor (ER); progesterone receptor (PR); human epidermal growth factor receptor (HER-2).

Discussion

Breast cancer is a highly heterogeneous tumor whose occurrence, metastasis, and prognosis vary in each patient. Therefore, individualized treatment of tumors is the development trend in research. The traditional clinical staging, prognosis, and treatment-related factors mainly include tumor size, histologic classification and grade, lymph node metastasis, and related molecular biologic indicators (ER, PR, HER-2, and Ki67) [11-13]. Since Ashworth et al. first discovered CTCs in peripheral blood of patients with advanced breast cancer in 1869, the mechanism of CTCs in the occurrence and development of breast cancer has been extensively studied. Relevant studies have shown that CTCs can be used to diagnose metastatic breast cancer, and can be used as an independent prognostic factor for patients with meta-

Table 5. Correlation analysis between CD24 positive circulating tumor cell detection results and clinical characteristics

Clinical characteristic		CD24 positive CTCs		
		≥ 1 (n = 60)	< 1 (n = 42)	*P*
Age (years)	≥ 50	26 (43.33)	19 (45.24)	0.849
	< 50	34 (56.67)	23 (54.76)	
Tumor site	Left	29 (48.33)	22 (52.38)	0.687
	Right	31 (51.67)	20 (47.62)	
Tumor size	≥ 3 cm	35 (58.33)	15 (35.71)	0.025
	< 3 cm	25 (41.67)	27 (64.29)	
Pathological type	Carcinoma in situ	1 (1.67)	1 (2.38)	0.307
	Micro infiltration	0 (0)	2 (4.76)	
	Infiltration	59 (98.33)	39 (92.86)	
T stage	T1	20 (33.33)	18 (42.86)	0.615
	T2	37 (61.67)	22 (52.38)	
	T3	3 (5.00)	2 (4.76)	
N stage	N0	19 (31.67)	23 (54.76)	0.098
	N1	26 (43.33)	10 (23.81)	
	N2	10 (16.67)	5 (11.91)	
	N3	5 (8.33)	4 (9.52)	
TNM stage	I	4 (6.67)	14 (33.33)	0.002
	II	39 (65.00)	17 (40.48)	
	III	17 (28.33)	11 (26.19)	
Molecular subtyping	Luminal A	5 (8.33)	6 (14.29)	0.400
	Luminal B HER-2 positive	5 (8.33)	7 (16.67)	
	Luminal B Her-2 negative	22 (36.67)	15 (35.71)	
	HER-2 positive	16 (26.67)	6 (14.28)	
	Triple-negative	12 (20.00)	8 (19.05)	
Lymphatic metastasis	with	41 (68.33)	19 (45.24)	0.020
	without	19 (31.67)	23 (54.76)	
ER expression	positive	33 (55.00)	28 (66.67)	0.237
	negative	27 (45.00)	14 (33.33)	
PR expression	positive	29 (48.33)	23 (54.76)	0.523
	negative	31 (51.67)	19 (45.24)	
HER-2 expression	positive	36 (60.00)	23 (54.76)	0.598
	negative	24 (40.00)	19 (45.24)	
Ki67 expression	high	20 (33.33)	9 (21.43)	0.190
	low	40 (66.67)	33 (78.56)	

Circulating tumor cells (CTCs); tumor node metastasis (TNM); estrogen receptor (ER); progesterone receptor (PR); human epidermal growth factor receptor (HER-2).

static breast cancer [14, 15]. However, the prognosis of different subtypes of CTCs in patients with early breast cancer is still unclear; therefore, this study evaluated the correlation of different subtypes of CTCs with clinical characteristics of breast cancer patients.

The results of this study showed that the positive detection rate of epithelial-CTCs was 62.74% (64/102); the positive detection rate of mesenchymal-CTCs was 45.10% (46/102); and the positive detection rate of mixed-CTCs was 73.53% (75/102). Mesenchymal-CTCs had no significant correlation with age, tumor location, tumor size, pathologic type, T stage, N stage, molecular type, lymph node metastasis, ER expression, PR expression, HER-2 expression, or Ki67 expression of breast cancer

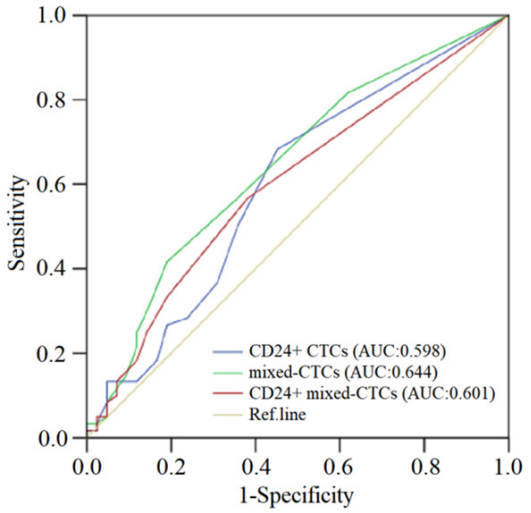

Figure 2. Receiver operator characteristic curve (ROC curve). The blue line is the number of CD24-positive CTCs, the green line is the number of mixed-CTCs, and the red line is the number of CD24-positive mixed-CTCs. Circulating Tumor Cells (CTCs).

patients. However, epithelial-CTCs were closely related to ER expression and TNM staging in breast cancer patients. Mixed-CTCs were closely related to lymph node metastasis of patients. These results suggest that the clinical characteristics of breast cancer patients may vary between different types of CTCs. Therefore, it is necessary to detect different types of CTCs in the analysis of the impact of CTCs on patient prognosis. Related studies have shown that epithelial-mesenchymal transition (EMT) enhances the migration and invasion of cancer cells [16]. In this study, we found that the type of epithelial-CTCs were closely associated with TNM stage and ER expression in breast cancer. However, there was no significant correlation between mesenchymal-CTCs and lymph node metastasis or tumor size; the reason may be because of the lower positive rate of interstitial CTC due to fewer samples and most patients were staged I-II. This study also found that mixed-CTCs (a mixture of epithelial and mesenchymal tumor cells) were closely associated with lymph node metastasis and larger tumor size, which were poor prognostic clinical features, suggesting that mixed-CTCs may promote tumor metastasis. On the one hand, mixed-CTCs can exhibit enhanced stem cell properties [17] and can freely convert to epithelial or mesenchymal types [18]. On the other hand, mixed-CTCs are more resistant to apoptosis [19] and are associated with chemoresistance [20].

CD24 is a highly glycosylated protein linked to the cell surface by glycosylphosphatidylinositol to facilitate downstream signaling networks, mainly expressed by immune cells but frequently overexpressed in human tumors. According to in vitro experiments with cancer cells, CD24 is a regulator of cell migration, invasion, and proliferation, its expression is associated with poor prognosis, and it is used as a cancer stem cell marker [21]. Therefore, we hypothesized that the expression of CD24 in CTCs was related to the prognosis of breast cancer, but there were no prior reports. In order to further verify the above conjecture, this study further analyzed the expression of CD24 in CTCs and the effect of CD24 expression on the survival of breast cancer patients. The results showed that the expression of CD24 in peripheral blood CTCs was significantly correlated with lymph node metastasis and tumor size, indicating that CD24 expression may reflect the malignant degree and proliferation and metastasis ability of tumors. In addition, the cumulative survival of patients with CD24 positive CTCs was significantly lower than that of patients with CD24 negative CTCs, indicating that its expression is associated with poor prognosis. CD24 expression has the same effect in some other malignant tumors. In ovarian cancer patients undergoing primary surgery, elevated CD24 expression is significantly associated with poor survival [22]. In another meta-analysis of breast cancer, the results showed that high expression of CD24 was significantly correlated with lower overall survival rate, lower disease-free survival rate, and some clinicopathologic factors (such as lymph node invasion and TNM stage), based on which it was concluded that CD24 was an effective marker of prognosis of breast cancer [23]. The results also further confirmed that CD24 was an efficient prognostic factor in breast cancer [23]. However, in this study, multivariate Cox regression analysis found that CD24-positive CTCs were not independent risk factors for prognosis of breast cancer patients. This may be partly related to the small sample size of this study, which should be increased in the future. Of note, the cumulative survival of patients with CD24-positive mixed-CTCs was significantly lower than that of patients without CD24-positive mixed-CTCs. Relevant studies have shown that EMT often manifests as incomplete activation of the invasion and metastasis cascade, as an intermediate stage of the invasion and metastasis cascade, and this

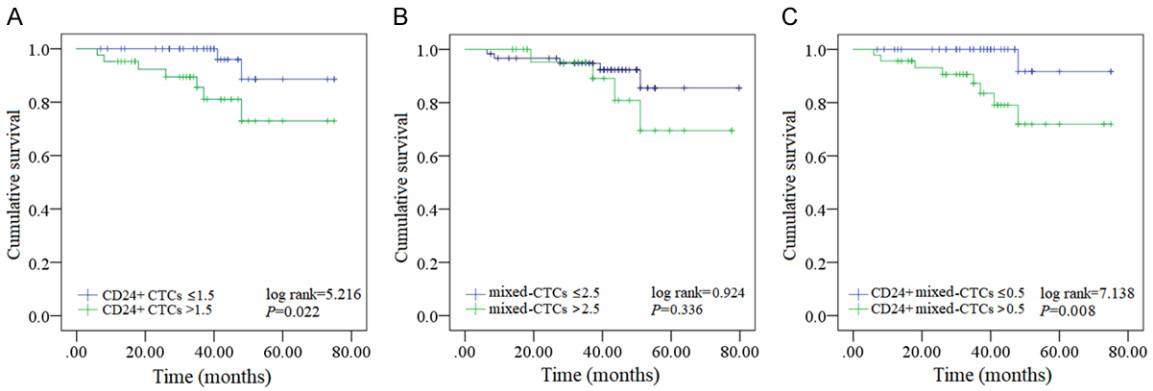

Figure 3. Survival analysis. A: CD24 positive total circulating tumor cells; B: Mixed circulating tumor cells; C: CD24 positive mixed circulating tumor cells.

Table 6. Cox regression analysis

	B	SE	Wald	P	Exp (B)	95% CI upper limit	95% CI lower limit
CD24 CTC	0.187	1.035	0.033	0.857	1.205	0.159	9.156
mixed-CTC	-0.355	0.706	0.253	0.615	0.701	0.176	2.798
CD24 mixed-CTC	2.309	1.382	2.790	0.095	10.061	0.670	151.037

Circulating tumor cells (CTCs).

subtype is called biophenotypic epithelial-mesenchymal. Biophenotypic can stimulate cell aggregation and survival in the blood circulation (mesenchymal) with partial preservation of intercellular adhesions (epithelial) [24]. Multicellular aggregation was observed in blood samples from breast cancer patients, and it was found that mixed-CTCs may promote cell cluster formation [25]. The ability of CTCs to form clusters is associated with increased metastatic potential [5, 26]. Based on the above results, we believe that CD24-positive CTCs, especially CD24-positive mixed-CTCs, may be one of the factors leading to poor prognosis of breast cancer patients. Therefore, in clinical practice, attention should be paid to these CD24-positive patients, and corresponding treatment measures should be given to improve survival.

Conclusion

The results of this study preliminarily show that mixed-CTCs are associated with lymph node metastasis in breast cancer patients. CD24-positive CTCs are associated with the prognosis and can be used as prognostic markers. Limitations include, first, that the study lacked the clinical data of normal controls; second, although all the included patients received surgical treatment, different postoperative treatment regimens may affect the prognosis to some extent. Therefore, the value of different types of CTCS in clinical characteristics remains to be supported by prospective, large-sample, multicenter clinical studies.

Acknowledgements

The work is financially supported by the Natural Science Foundation of Hunan Province, General Project (2019JJ40452).

Disclosure of conflict of interest

None.

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