

Role of regulatory T cells and CD8⁺ T lymphocytes in the dissemination of circulating tumor cells in primary invasive breast cancer

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Received November 24, 2017; Accepted May 4, 2018

DOI: 10.3892/ol.2018.8993

Abstract. Circulating tumor cells (CTCs) dissemination is involved in tumor metastasis and is an independent prognostic factor in patients with primary and metastatic breast cancer. Regulatory T cells (Tregs) and cluster of differentiation (CD)8⁺ T lymphocytes are the main types of immune cells in the tumor microenvironment and exert opposite roles on the progression and outcome of breast cancer. The aim of the present study was to evaluate the associations between CTCs and intratumoral/peritumoral Tregs and CD8⁺ T lymphocytes in breast cancer. Peripheral CTCs were detected by a multi-marker quantitative polymerase chain reaction platform in 167 patients with invasive breast cancer. Intratumoral/peritumoral Tregs and CD8⁺ T lymphocytes were assessed by immunohistochemistry in 167 patients with invasive breast cancer to establish an association between these cell types and detection of peripheral CTCs. CTCs were detected in 55% of the patients with breast cancer. The prevalence of CTCs was positively associated with the number of intratumoral (P=0.002) and peritumoral Tregs (P=0.045), and the primary tumor size (P=0.012). This result was verified by analyzing intratumoral Tregs (P=0.044) and primary tumor size (P=0.044) with multivariate analysis, which indicated that the CTC-positive rate increased with an increasing number of intratumoral Tregs and a larger tumor size. In the multivariate analysis, other variables including menopause, tumor-node-metastasis stage and peritumoral Tregs were not associated with the prevalence of CTCs. The

prevalence of CTCs was inversely and weakly associated with the number of peritumoral CD8⁺ T lymphocytes using the univariate analysis, however this result was not statistically significant (P=0.470). In conclusion, regulatory T cells and CD8⁺ T lymphocytes may be involved, at least in part, in the CTCs dissemination of breast cancer, whereby Tregs appear to exert the dominant effect. Furthermore, the role of Tregs in the progression of breast cancer may be mediated by suppressing the dissemination of CTCs, which is primarily determined by intratumoral Tregs.

Introduction

Metastasis is a major cause of mortality in breast cancer. The metastatic cascade is composed of a series of biological steps that enables tumor cells to detach from the primary tumor, invade into the bloodstream and lymphatic system, extravasate into the parenchyma and establish a new metastatic lesion (1). This process may occur at the early stages of breast cancer. Circulating tumor cells (CTCs) may have a crucial role in the metastatic cascade and are a prognostic factor in patients with primary and metastatic breast cancer (2). CTC dissemination, as a part of metastasis, is a highly inefficient process, and only a minority of tumor cells that escape from local and systemic immune surveillance are capable of forming metastases (3-5). Therefore, immune changes in the primary tumor microenvironment may be involved in the dissemination of CTCs in breast cancer. Current evidence concerning the association between CTCs and the immune system in breast cancer is limited (6,7).

Tumor-infiltrating lymphocytes (TILs) are heterogeneous immune cells infiltrating in the tumor microenvironment and are considered to be the main components of the body's immune surveillance mechanism against cancer. In breast cancer, TILs are a heterogeneous population that may promote humoral and cellular antitumor responses (3,8). For example, as the predominant T lymphocytes infiltrate the tumor microenvironment, cluster of differentiation CD8⁺ T lymphocytes may exert cytotoxic activity towards tumor cells by triggering apoptosis (9). Therefore, CD8⁺ T lymphocytes

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Key words: breast cancer, circulating tumor cells, regulatory T cells, cluster of differentiation 8⁺ T lymphocytes

are hypothesized to be the frontline defense against cancer. Several studies have observed an association between a high number of CD8⁺ TILs and favorable clinical outcomes (10,11). When CD8⁺ T lymphocytes fail to eliminate tumor cells, these tumor cells, which have reduced immunogenicity, may break through the primary tumor microenvironment (12). Accordingly, poor infiltration of CD8⁺ T lymphocytes may have a role in the initial dissemination of CTCs. Apart from a decrease in immune surveillance, several subsets of TILs are known to suppress anti-tumor immunity (3), which may participate in the dissemination of CTCs.

Considerable attention has been paid to regulatory T cells (Tregs), which have been identified as forkhead box (Foxp3)⁺ T cells (12,13). Tregs have an important role in immune evasion in cancer cells (14,15). Studies have confirmed that an increased number of intratumoral or peritumoral Tregs has a negative impact on the prognosis of breast cancer depending on the localization in the tumor microenvironment (16-18). Due to their opposing actions in tumor immunity and established use for breast cancer prognosis, a combined assessment of CD8⁺ T lymphocytes and infiltration of Tregs may be used to assess the impact of the immune microenvironment on the dissemination of CTCs in breast cancer (19-21).

The present study was conducted to assess the association between the number of tumor infiltrating CD8⁺ T lymphocytes/Tregs and the presence of CTCs in the peripheral blood. The TILs analyzed in the present study include the intratumoral and peritumoral cell types. A multi-marker quantitative polymerase chain reaction (qPCR) platform was established by using a combination of three widely used mRNA markers: Cytokeratin 19 (CK19), human mammaglobin (hMAM) and small breast epithelial mucin (SBEM) (21,22). In addition, the infiltrating CD8⁺ lymphocytes and Tregs were detected using antibodies against CD8 and Foxp3 by immunohistochemistry (IHC). Then, the association between the prevalence of CTCs and intratumoral/peritumoral Tregs or CD8⁺ T lymphocytes was analyzed.

Materials and methods

Patients. Between May 2015 and January 2017, 167 patients with invasive breast cancer, who were treated at the Department of Thyroid and Breast Surgery of The Affiliated Hospital of Nantong University (Nantong, China), were enrolled in the present study. The median age of the patients was 50 years (range, 26-84 years). The clinicopathological parameters evaluated for each patient included age, menstrual status, tumor size, tumor-node-metastasis (TNM) stage, histological grade, estrogen receptor (ER) and progesterone receptor (PR) status and human epidermal growth factor receptor 2 (HER2) status (Table I). For each patient, a detailed past medical history was recorded to exclude liver and kidney dysfunction, autoimmune disease, bone marrow disease and chronic infection. All tissue and blood samples were collected prior to chemotherapy, radiotherapy, endocrine therapy or any other treatment that could affect the immune state. A complete diagnostic evaluation, including history, physical examination, ultrasound, chest computed tomography (CT), abdominal CT, bone scan and tumor marker tests, was performed to exclude distant metastasis.

To define the baseline expression and positive threshold of the three markers (CK19, hMAM and SBEM), peripheral blood samples were also collected from control subjects. A total of 30 healthy female volunteers (aged 23-85 years; median, 44 years) and 60 patients with pathologically diagnosed benign breast disease (aged 23-72 years; median, 38 years) were enrolled between May 2015 and January 2017. The present study was approved by the Ethics Committee of Nantong University (Nantong, China) and The Affiliated Hospital of Nantong University (Nantong, China). All donors and patients provided written informed consent.

Blood processing. Following shedding of the first 2 ml of peripheral blood to reduce contamination by epithelial cells from the skin, 10 ml peripheral blood was collected in an EDTA-treated vacuum tube from each patient or control subject. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood by Ficoll density gradient centrifugation (Ficoll-Paque Plus; TBDscience, Tianjin, China) using standard procedures and frozen at -80°C until RNA extraction.

Reverse transcription-qPCR. Total RNA was isolated from PBMCs of patients with invasive breast cancer control subjects and 4 breast cancer tissue specimens. These were selected as breast cancer tissues have been proven to express the genes CK19, hMAM and SBEM, it was chosen to verify the stability of the multi-marker RT-PCR system, and served as a positive control. Total RNA was isolated using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The purity and quantification of RNA were assessed by measuring the optical density at 260 and 280 nm. The RNA quality was also verified by electrophoresis in a 1.5% non-denaturing agarose gel. The RNA integrity was assessed by qPCR amplification of the housekeeping gene, β -actin. Reverse transcription of the RNA was performed using the Prime-Script RT Master Mix system (Takara Biotechnology Co., Ltd.). cDNA was synthesized from 1.5 mg total RNA that was isolated from PBMCs in a total volume of 30 μ l according to the manufacturer's protocol.

The primers for CK19, SBEM, hMAM and β -actin were designed using the software Primer version 5.0 (PREMIER, Palo Alto, CA, USA). All primers were synthesized by Invitrogen (Table II) (Invitrogen; Thermo Fisher Scientific, Inc.). All PCR reactions were performed using the ABI StepOnePlus real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Inc.) and fluorescent SYBR-Green I. qPCR was performed using the SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd.) according to the protocol in a final volume of 20 μ l containing 10 μ l 2X SYBR Premix Ex Taq, 0.4 μ l PCR forward primer (10 μ M), 0.4 μ l PCR reverse primer (10 μ M), 0.4 μ l 50X ROX reference dye, 2 μ l cDNA template and 6.8 μ l dH₂O. The thermocycling conditions consisted of 30 sec of predenaturation at 95°C and 40 cycles of PCR (denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec).

The relative expression of the marker genes was normalized to β -actin expression and calculated using the formula: $2^{-\Delta\Delta C_q}$ (23). The relative gene expression value for the marker genes (CK19, hMAM and SBEM), in the peripheral blood of a healthy volunteer was set to 1.

Table I. Association between the CTC-positive rate in the peripheral blood of patients with breast cancer and clinicopathological parameters.

Clinicopathological parameters	Total, n=167	CTC-positive rate (%), n=92	CTC-negative rate (%), n=75	P-value
Age, years				0.530
≤45	45	23 (51)	22 (49)	
>45	122	69 (57)	53 (43)	
Menopausal status				0.084
Premenopausal	68	32 (47)	36 (53)	
Postmenopausal	99	60 (61)	39 (39)	
Tumor size ^a				0.012
T1	65	27 (42)	38 (58)	
T2	98	62 (63)	36 (37)	
T3	4	3 (75)	1 (25)	
Lymph node stage ^a				0.120
pN0	102	52 (51)	50 (49)	
pN1	34	20 (59)	14 (41)	
pN2	15	7 (47)	8 (53)	
pN3	16	13 (81)	3 (19)	
Disease stage ^a				0.095
I	43	18 (42)	25 (58)	
II	92	53 (58)	39 (42)	
III	32	21 (66)	11 (34)	
Histological grade				0.494
1	12	6 (50)	6 (50)	
2	98	51 (52)	47 (48)	
3	57	35 (61)	22 (39)	
ER/PR status ^b				0.600
Negative	50	26 (52)	24 (48)	
Positive	117	66 (56)	51 (44)	
HER2 status ^c				0.143
Negative	127	65 (51)	62 (49)	
Positive	40	27 (67)	13 (33)	

^aClassified according to the Union for International Cancer Control. ^bER/PR status was determined to be positive when the expression of one or both of the receptors are positive. ^cHER2 status was determined to be positive when a score of 3+ was attained in immunohistochemistry, or positive fluorescence was detected by *in situ* hybridization. CTC, circulating tumor cell; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

qPCR positivity was defined as gene expression beyond the cut-off threshold, which was set for each gene marker at three standard deviations from the mean expression in the control subjects (24). Positivity of the multi-marker method was defined as positivity for at least one of the markers.

IHC staining and quantification of CD8 and Foxp3. Formalin-fixed (concentration 10%, room temperature, fixed for 24 h) paraffin sections (4 μm) of the tumor tissues were subjected to IHC staining. Briefly, the sections were deparaffinized and rehydrated in graded alcohol. Antigen retrieval was performed at 121°C for 2 min in citrate buffer (pH 6.0). After serial blocking with hydrogen peroxide and normal goat serum (Luoshen Biotechnologies, Inc., Shanghai, China,

http://eastdiagno.bioon.com.cn/index_93476.html) at room temperature for 10 min, the sections were incubated with a primary monoclonal antibody against CD8 (catalog no, MA5-13473; clone C8/144B; dilution, 1:40; Thermo Fisher Scientific, Inc.) or Foxp3 (catalog no, ab20034; clone 236A/E7; dilution, 1:100; Abcam, Cambridge, UK) overnight at 4°C. Labeling was detected using the PV-6000 Polymer Detection system (OriGene Technologies, Inc., Beijing, China). Sections were counterstained with hematoxylin at room temperature for 3 min. Positive staining controls were performed in parallel with tonsil sections provided by patients in otolaryngology between October 2016 and January 2017, including 2 males and 3 females. A negative staining control was performed by omitting the use of the primary antibody.

Table II. Prime sequences.

Gene	Sequence (5'-3')
CK19	
Sense	TCCGAACCAAGTTTGAGACG
Antisense	CCCTCAGCGTACTGATTTCTT
hMAM	
Sense	AGAACTGCAGGGTATGGTGAGAA
Antisense	ACATGTATAGCAGGTTTCAACAATTGT
SBEM	
Sense	GATCTTCAGGTCACCACCATG
Antisense	GGGACACACTCTACCATTCTG
β -actin	
Sense	GCTGTGCTATCCCTGTACGC
Antisense	TGCCTCAGGGCAGCGGAACC

CK19, cytokeratin 19; hMAM, human mammaglobin; SBEM, small breast epithelial mucin.

The CD8⁺ T lymphocytes and Foxp3⁺ Tregs were quantified independently by two trained investigators, who were blinded to the clinical data and positivity of peripheral CTCs. A total of 5 intratumoral and 5 peritumoral areas were selected and imaged using an x10 objective lens of a light microscope (Nikon 50i and Nikon DS-U3; Nikon Corporation, Tokyo, Japan). The images were obtained using an x40 objective lens for cell counting. Due to the extensive heterogeneity in peritumoral infiltrating CD8⁺ T lymphocytes and Foxp3⁺ Tregs, a modified grading system for semi-quantitative scoring was used. In this system, G0 corresponded to the absence of lymphocytes. G1 corresponded to the mean number of CD8⁺ T lymphocytes ≤ 50 and Foxp3⁺ Tregs ≤ 25 . G2 corresponded to the mean number of CD8⁺ T lymphocytes > 50 and ≤ 100 and Foxp3⁺ Tregs > 25 and ≤ 50 . G3 corresponded to the mean number of CD8⁺ T lymphocytes > 100 and Foxp3⁺ Tregs > 50 .

Statistical analysis. The associations between variables were evaluated by Pearson's χ^2 test or Fisher's exact test if required. A univariate analysis was used to test if there are associations between CTC-positive rate and the number of Tregs, CD8⁺ T lymphocytes and clinicopathological parameters. A multivariate logistic regression model was also

performed to analyze the independent factors associated with the CTC-positive rate. Statistical analysis was performed using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). In all analyses, a two-sided $P < 0.05$ was considered statistically significant.

Results

Multi-marker qPCR analysis of CTCs in patients with breast cancer. The marker gene expression baseline was precisely quantified in 90 control subjects by qPCR. The median relative gene expression values for CK19, hMAM and SBEM in the control subjects were 1.18, 1.92 and 1.29, respectively. The cut-off threshold for marker gene positivity, that is, abnormal expression, was set at three standard deviations from the mean $2^{-\Delta\Delta C_q}$ value for each gene. The cut-off thresholds for CK19, hMAM and SBEM were 4.92, 7.71 and 6.38, respectively. Of 167 patients, 62 (37%) were positive for CK19, 37 (22%) were positive for hMAM, and 40 (24%) were positive for SBEM (Table III). In total, 92 (55%) patients were positive for at least one marker. The sensitivities for the individual markers CK19, hMAM, and SBEM were 37, 22 and 24%, respectively, and the specificities were 100, 99 and 99%, respectively. The multi-marker qPCR platform yielded a sensitivity of 55% and a specificity of 98% (Table III).

Detection of CD8⁺ T lymphocytes and Foxp3⁺ Tregs in the tumor microenvironment of breast cancer. Infiltrating intratumoral CD8⁺ T lymphocytes and Foxp3⁺ Tregs presented with a diffuse pattern, and the peritumoral infiltration was more abundant and tended to form lymphoid aggregates (Fig. 1). There were significantly more CD8⁺ T lymphocytes (median, 79.03 vs. 25.00; range, 0-254.11 vs. 0-138.67) and Foxp3⁺ Tregs (median, 34.12 vs. 10.33; range 0-188.03 vs. 0-85.67) in the peritumoral than the intratumoral areas (data not shown).

Association between CTC-positive rate and the clinicopathological parameters of patients with breast cancer. The peripheral CTC-positive rate was statistically significant between the different tumor sizes ($P < 0.05$) and was associated with a larger tumor size (T1, 42%; T2, 63%; T3, 75%) (Table I). The peripheral CTC-positive rate was also weakly associated with a higher disease stage and histological grade, although this association did not reach significance ($P > 0.05$). Associations between CTC-positive rate and the remaining clinicopathological parameters were not observed ($P > 0.05$; Table I).

Table III. CTC-positive rate in controls and patients with breast cancer.

Groups	CTC-positive rate of three marker genes (%)	CTC-positive rate of individual marker genes (%)		
		CK19	hMAM	SBEM
Control (n=90)	2/90 (2.2)	0/90 (0.0)	1/90 (1.1)	1/90 (1.1)
Breast cancer (n=167)	92/167 (55)	62/167 (37)	37/167 (22)	40/167 (24)

CK19, cytokeratin 19; hMAM, human mammaglobin; SBEM, small breast epithelial mucin.

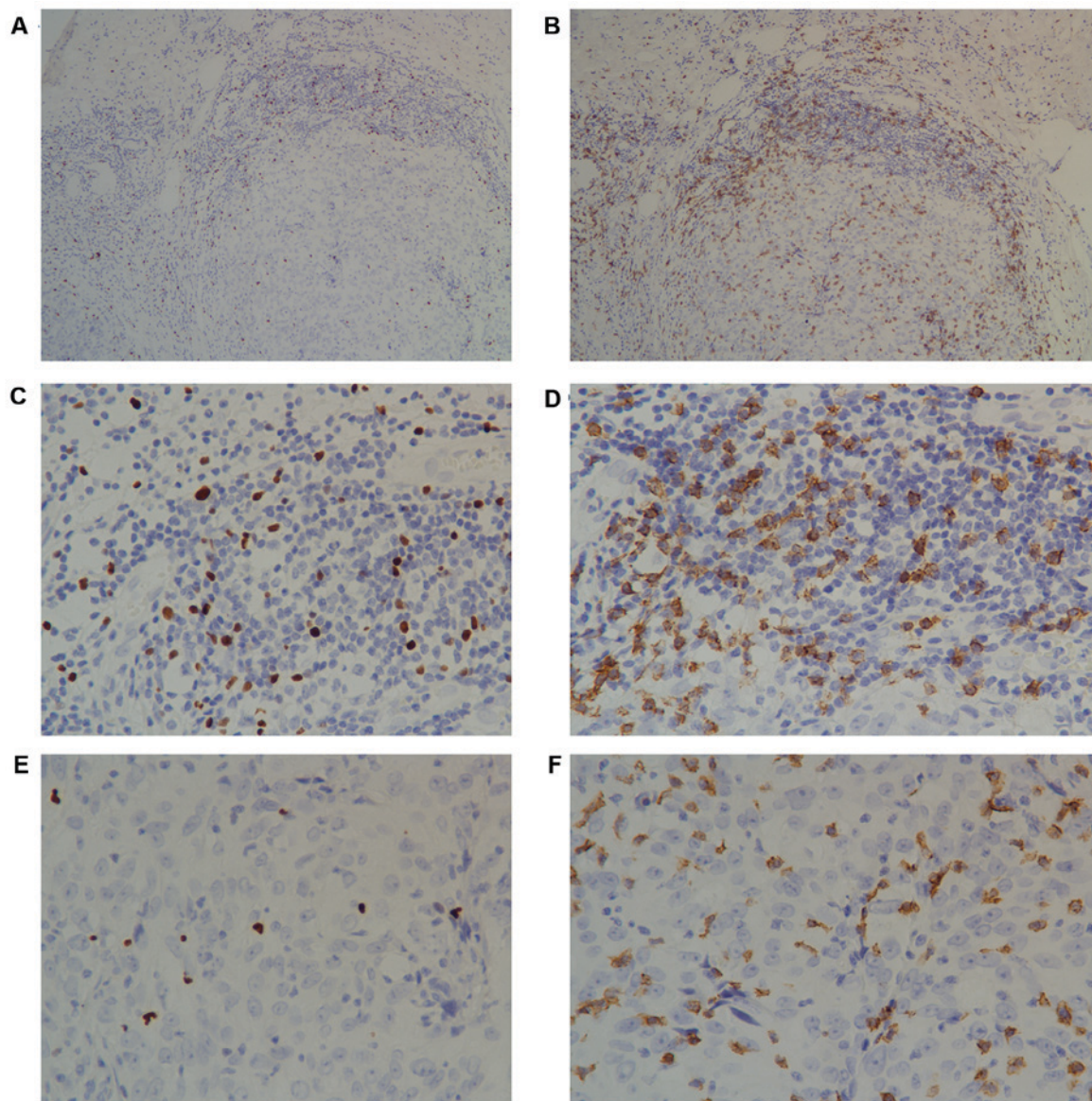


Figure 1. Immunohistochemical analyses of intratumoral and peritumoral lymphocytes. Immunohistochemical staining of tumor-infiltrating (A) Foxp3⁺ T cells and (B) CD8⁺ T lymphocytes in invasive breast cancer. A diffuse pattern of intratumoral lymphocytes was observed. By contrast, peritumoral lymphocytes formed a lymphoid aggregate (x10 objective lens). There were markedly more Foxp3⁺ Tregs in the (C) peritumoral area than the (E) intratumoral areas. There was an increased number of CD8⁺ lymphocytes in the (D) peritumoral area than the (F) intratumoral areas. A and B, x10 objective lens; B-E, x40 objective lens. CD, cluster of differentiation; Foxp3, forkhead box 3.

Association of CTC-positive rates with Foxp3 T cells but not with CD8⁺ T cells in the tumor microenvironment. It was determined that increased numbers of intratumoral Tregs are associated with the CTC-positive rate in patients with breast cancer (Table IV). Patients with an increased number of intratumoral Tregs exhibited higher CTC-positive rates compared with those with a decreased number of Tregs (67 vs. 43%, $P=0.002$; Fig. 2). The same trend was observed for peritumoral infiltrating Tregs (G0, 48%; G1, 42%; G2, 57%; G3, 73%, $P=0.045$) (Table IV). Detection of peripheral CTCs was weakly and inversely associated with the number of infiltrating peritumoral CD8⁺ lymphocytes, although this association did not reach statistical significance ($P=0.47$; Table IV).

Multivariate analysis indicated that the prevalence of CTCs was independently associated with tumor size (OR=2.18,

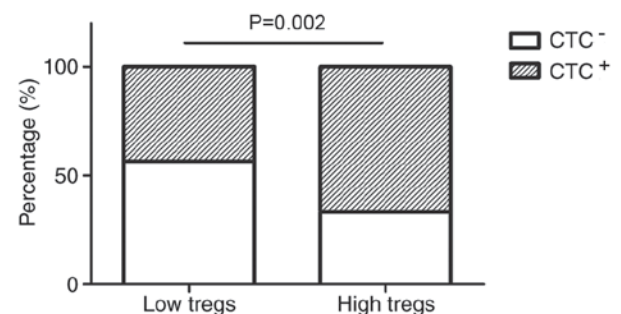


Figure 2. Association between CTCs in the peripheral blood of patients with breast cancer and infiltrating intratumoral Foxp3⁺ Tregs. The patients with breast cancer were divided into two groups according to the median (10.33). Patients with an increased number of intratumoral Foxp3⁺ Tregs exhibited a higher CTC-positive rate compared with those with a lower number of Foxp3⁺ Tregs (67 vs. 43%, $P=0.002$). CTC, circulating tumor cells; Tregs, regulatory T cells; Foxp3, forkhead box 3.

Table IV. Association between the CTC-positive rate in the peripheral blood of patients with breast cancer and tumor-infiltrating CD8⁺ T lymphocytes and Foxp3⁺ Tregs in the tumor microenvironment.

Infiltration of lymphocytes	CTC-positive rate (%)	CTC-negative rate (%)	P-value
Intratumoral Foxp3 ⁺ Tregs ^a			0.002
Low	36 (43)	47 (57)	
High	56 (67)	28 (33)	
Intratumoral CD8 ⁺ T lymphocytes ^a			0.846
Low	44 (54)	37 (46)	
High	48 (56)	38 (44)	
Peripheral Foxp3 ⁺ Tregs ^b			0.045
G0	24 (48)	26 (52)	
G1	14 (42)	19 (58)	
G2	27 (57)	20 (43)	
G3	27 (73)	10 (27)	
Peripheral CD8 ⁺ T lymphocytes ^b			0.470
G0	17 (65)	9 (35)	
G1	22 (59)	15 (41)	
G2	23 (55)	19 (45)	
G3	30 (48)	32 (52)	

^aMedium level was used as a cut-off (Foxp3⁺ Tregs, 10.33; and CD8⁺ T lymphocytes, 25.00). ^bG0 corresponded to the absence of lymphocytes. G1 corresponded to the mean number of CD8⁺ T lymphocytes ≤50 and Foxp3⁺ Tregs ≤25. G2 corresponded to CD8⁺ T lymphocytes >50 and ≤100 and Foxp3⁺ Tregs >25 and ≤50. G3 corresponded to CD8⁺ T lymphocytes >100 and Foxp3⁺ Tregs >50. CD, cluster of differentiation; Foxp3, forkhead box 3; Tregs, T regulatory cells.

Table V. Multivariate analysis of the association between circulating tumor cells in the peripheral blood of breast cancer patients and clinicopathological parameters as well as Foxp3⁺ Tregs^a.

Variables	OR	95% CI	P-value
Menopausal status	0.49	0.25-0.98	0.052
Tumor size	2.18	1.02-4.64	0.044
TNM stage	1.17	0.64-2.14	0.608
Intratumoral Tregs	2.23	1.02-4.87	0.044
Peritumoral Tregs	1.16	0.83-1.60	0.369

^aParameters with P-value ≤0.1 in univariate analysis were selected for multivariate analysis. CI, confidence interval; Foxp3, forkhead box 3; OR, odds ratio; TNM, tumor node metastasis; Tregs, T regulatory cells.

95% CI, 1.02-4.64; P=0.044) and infiltrating intratumoral Tregs (OR=2.23, 95% CI, 1.02-4.87; P=0.044) (Table V).

Discussion

The association between CTCs and the immune system in breast cancer was reported (25,26). The effect of TILs on the prevalence of CTCs in patients with breast cancer is poorly understood. In the present study, it was demonstrated that

the number and distribution of tumor-infiltrating Tregs were significantly associated with the presence of peripheral CTCs in patients with breast cancer.

CTCs can be identified and characterized in the peripheral blood of patients with breast cancer (27) and may represent the emergence of early hematological metastasis in breast cancer. CTCs have the ability to form clusters of microscopic tumors, and their detection, persistence or elevation is associated with poor prognosis (2). Therefore, it is essential to elucidate the factors that affect the dissemination of CTCs, which would provide a theoretical basis for improving breast cancer treatment. Immune cells, cytokines and other factors in the microenvironment are present throughout tumor development and have an impact on tumor progression (28); therefore, the effects of immune factors on disease progression may be partially mediated by the suppression of hematological micro-metastasis in breast cancer.

Due to the heterogeneity of tumor cells and the instability of gene expression, there is no identified and specific standard for CTCs detection to date (29). Our center has established a multi-marker (CK19, hMAM, SBEM) qPCR platform for detection of CTCs in the peripheral blood of patients with breast cancer. The sensitivity and specificity of this method have been confirmed in several laboratories (30,31). CK19, hMAM and SBEM mRNA were detected in 37, 22 and 24% of the patients with breast cancer, respectively. When using the multi-marker platform (for which positivity was defined as positivity for at least one of the markers), the sensitivity was improved to 55%, which was greater compared with the values

reported in similar studies (31,32). In addition, the specificity of the multi-marker platform was 98% due to contamination of only minor samples. Therefore, the multi-marker qPCR platform provides high sensitivity and specificity for the detection of CTCs.

TILs exert an important role in tumor immunity. Tregs were initially characterized as CD4⁺ CD25⁺ cells and are thought to be the main obstacles that hinder the efficacy of antitumor immunity and immunotherapy (30,33). Tregs effectively suppress the proliferation and activation of CD8⁺ T lymphocytes in a contact-dependent manner or via the release of cytokines (33). The impact of Tregs on the prognosis of breast cancer depends on their distribution and the expression of hormone receptors (34). The majority of studies have reported that an elevated number of intratumoral/peritumoral Tregs confers a poor clinical outcome (16-19) and is associated with disease progression in breast cancer (34). However, West *et al* (35) concluded that Tregs exhibit an antitumor activity as it may favor the survival time of patients with ER-negative breast cancer. Therefore, the present study investigated the association between the number of intratumoral/peritumoral Tregs and the presence of peripheral CTCs. Univariate analysis demonstrated that patients with an increased number of intratumoral/peritumoral Tregs exhibited a higher CTC-positive rate compared with those with a lower number of Tregs. Therefore, increasing the number of intratumoral/peritumoral Tregs may increase local immunosuppressive capabilities and reduced anti-tumor immunity, consequently forming an immunosuppressive microenvironment conducive to tumor growth, invasion and metastasis.

A local antitumor immunity deficiency may indicate a depressed systemic immune system (36,37). A depressed immunity in the tumor microenvironment may inefficiently stimulate tumor-antigen presentation or the immune response, which would enable breast cancer cells to escape from local or systemic immune surveillance and potentially form distant metastases (34). Previous studies regarding the prognostic value of Tregs in breast cancer were consistent with the results of the present study (16-19). In addition, the multivariate analysis in the present study demonstrated that an increasing number of intratumoral Tregs but not peritumoral Tregs was an independent risk factor for detection of CTCs. The prognosis data from Liu *et al* (19) supported the present findings that an increased number of intratumoral Tregs but not peritumoral Tregs was associated with a poorer clinical outcome (16,19). However, the present results were different from those reported by Mahmoud *et al* (17). One explanation for these contradictory results may be the tissue volume used (19). The tissue array used by Gobert *et al* (18) contained only a small volume of tumor tissue. The use of such small tissue fragments may not be optimal to obtain a sufficient number of cells for a reliable count of Tregs in different areas. In the present study full block tissue sections were used to enable the selection of areas with abundant intratumoral/peritumoral Tregs.

Rich infiltration by CD8⁺ T lymphocytes, which are a representative subset of lymphocytes that are associated with immune surveillance in the tumor microenvironment, may indicate strong anti-tumor immunity (37). The results of the present study revealed that an elevated grade for peritumoral CD8⁺ T lymphocytes was inversely associated with the detection of

peripheral CTCs, although this association did not reach statistical significance. The hypothesis that CD8⁺ T lymphocytes suppress the dissemination of CTCs was not confirmed in the present study. This may be due to the following factors. First, either by a hematological or lymphangial route, breast cancer cells may invade the circulatory system and cause distant metastases. In the present study, the function of local immune suppression was examined in hematological micrometastasis rather than via the lymphangial route. In a study on human colorectal carcinoma, Chiba *et al* (38) observed that the effects of CD8⁺ T lymphocytes may be mediated by the suppression of lymphangial micrometastasis rather than growth suppression in the primary tumor. Secondly, the effect of CD8⁺ T lymphocytes on tumor progression depends not only on their number and distribution but also on their activity (3,39). Cytotoxic CD8⁺ T cells, which are merely active CD8⁺ T lymphocytes, may exert anti-tumor immunity. Although a significant association between tumor-infiltrating CD8⁺ T lymphocytes and the detection of peripheral CTCs was not observed, the possibility of this association cannot be ignored. Accumulating studies indicate that CD8⁺ T lymphocytes have antitumor activity, as they may favor the survival time of patients with breast cancer (9). Therefore, it is possible that CD8⁺ T lymphocytes may contribute to patient survival time via other mechanisms, and thus further research is required.

In conclusion, the results of the present study indicated that the role of Tregs in the progression of breast cancer may be mediated by the suppression of dissemination of CTCs, and the effect is mainly determined by intratumoral Tregs. Nevertheless, the impact of the phenotypic and functional qualities of Tregs or CD8⁺ T lymphocytes on hematological micrometastasis in breast cancer was not assessed comprehensively. Prospective studies are required to understand better the molecular mechanisms by which TILs affect the prevalence of CTCs and their potential role in breast cancer immunotherapy.

Acknowledgements

Not applicable.

Funding

The research was supported by The Nantong demonstration project for the people's livelihood (grant no. MS32015029).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

DX analyzed the data and was a major contributor in writing the manuscript. TX made substantial contribution in analysis and interpretation of the data. JW performed immunohistochemistry and cell counting. MC collected the information regarding the subjects enrolled in the present study and detected CTCs. SW had major role in designing the study. CZ designed the study and gave the final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nantong University (Nantong, China) and The Affiliated Hospital of Nantong University (Nantong, China). All donors and patients gave informed consent.

Patient consent for publication

Informed consent was obtained from all patients included in this study for publication of the associated data.

Competing interests

The authors declare that they have no competing interests.

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