

RESEARCH PAPER



Tumor-infiltrating Treg, MDSC, and IDO expression associated with outcomes of neoadjuvant chemotherapy of breast cancer

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ABSTRACT

Background: Regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) represent two immunosuppressive cell populations that are important in the establishment and maintenance of cancer immune tolerance. MDSCs can express IDO and promote immune tolerance via expansion of Treg cell.

Method: We use needle biopsy breast cancer tissues prior to neoadjuvant chemotherapy (NCT) staining for CD33, Foxp3 and IDO by immunohistochemistry to evaluate whether they were correlated with subsequent treatment responses in breast cancer.

Results: Expressions of IDO, CD33⁺MDSCs and Foxp3⁺Tregs were correlated with each other. Immunohistochemical double staining revealed that IDO expression in CD33⁺MDSCs was positively correlated with Foxp3⁺Tregs ($P < 0.05$). CD33⁺MDSCs, Foxp3⁺Tregs, and IDO expression in tumor tissues were associated with advanced clinical stage prior to NCT ($P < 0.05$). CD33⁺MDSCs, Foxp3⁺Tregs, IDO expression, IDO expression in CD33⁺MDSCs and clinical T3–T4 stage prior to NCT, pathological T3–T4 stage, ER(+), luminal type were correlated with clinical responses of PD+SD ($P < 0.05$). Multivariate analysis showed that CD33⁺MDSCs, IDO expression, IDO expression in CD33⁺MDSCs, and advanced pathological T stage were risk factors for PD+SD. Focusing on the pCR of NCT, only CD33⁺MDSCs, clinical T3–T4, and N1–N3 stage prior to NCT were associated with no-pCR ($P < 0.05$). The multivariate analysis showed that advanced clinical T stage and N stage were risk factors for no-pCR. Clinical stage prior to NCT were significantly correlated with progression free survival ($P = 0.021$), while Foxp3⁺Tregs and clinical T stage were significantly correlated with overall survival ($P = 0.022$ and $P = 0.001$, respectively). Foxp3⁺Treg was significant risk factor for overall survival after adjusting covariates by COX regression.

Conclusion: Tumor-infiltrating MDSCs, Tregs, IDO expression and IDO expression in MDSCs were correlated with clinicopathological features, NCT response, and prognosis of breast cancer patients, suggesting that they might be potential markers for clinical outcomes of NCT and help clinical decision-making for improved therapies for breast cancer.

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Introduction

The breast cancer treatment decision making depends on various factors, including disease stage, patient age hormone receptor etc. For patients with poor prognosis and high recurrence risk, more aggressive or radical treatments are proposed. Patients diagnosed with high-risk non-metastatic breast cancer, including locally advanced and inflammatory breast cancer, are usually treated with upfront chemotherapy, in the form of neoadjuvant chemotherapy (NCT) followed by surgery. This treatment has been demonstrated to decrease tumor burden and increase breast conservation rate.^{1,2} Simultaneously, as the response to NCT can be evaluated, there is possibility to modify regimens to increase rates of pathological complete response (pCR); thus, the primary tumor response serves as an *in vivo* chemosensitivity test²; therefore, this treatment has wide applications.

Regulatory T cell (Tregs) and myeloid derived suppressor cells (MDSCs) represent two immunosuppressive cell populations that play an important role in the establishment and maintenance of tumor immune escape. MDSCs are heterogeneous cells composed of myeloid cells blocked at many steps in differentiation. These cells were observed to be enriched in the peripheral blood, bone marrow, tumor tissues, lymphatic drainage areas, and cancer metastases of cancer patients.^{3–6} These cells are characterized by their ability to inhibit both innate and adaptive immune reactions, thus exerting a suppressive effect on antitumor immunity. In humans, markers for MDSCs currently are not unified until now. However, these cells are proposed characterized by Lin[−]CD33⁺CD11b⁺HLA-DR[−] recently.⁷ In our previous study, we found that the MDSCs characterized by CD45⁺CD13⁺CD33⁺CD14[−]CD15[−] were significantly increased in blood and tumor tissues,⁸ whereas *in vitro*, MDSCs

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characterized by CD45⁺CD13⁺CD33⁺CD14⁻CD15⁻ could be induced by MDA-MB-231 breast cancer cells when co-cultured with CD33⁺ myeloid cells from healthy donors.

Tumor-infiltrating Tregs stably express the transcription factor fork head box protein 3 (Foxp3) and down-regulate immune responses, which is often associated with poor clinical outcomes.⁹ In patients with malignant tumors, increased level of peripheral Tregs before therapy has been reported to be correlated with shorter progression free survival (PFS), whereas elevated Treg in the blood and tumor tissues of patients with ovarian carcinoma, non-small cell lung carcinoma (NSCLC), and hepatocellular carcinoma are associated with poorer prognosis and an higher recurrence risk.¹⁰⁻¹²

The mechanisms by which tumors promote the expansion and/or function of these suppressive cells and cross-talks between them are remain unclear. Recent studies demonstrated a distinct correlation between indoleamine 2, 3-dioxygenase (IDO), a rate-limiting enzyme of tryptophan catabolism, and tumor-induced immunosuppression.^{13,14} It was reported that MDSCs could promote immunosuppression via Treg expansion which is induced by IDO expression in MDSCs.¹⁵ It was reported that IDO exerts its suppressive effects through a reduction in local tryptophan and the generation of cytotoxic metabolites at tumors and lymphatic drainage areas to block the initiation of Ag-specific immune responses, the antitumor cytotoxicity of activated T cells, and to increase Tregs.¹⁶⁻¹⁸

In our previous study of breast cancer, we observed increased IDO expression in tumor tissues, which was associated with increased Foxp3⁺Treg cells in tumor situ and metastatic lymph nodes. Further, IDO was essential for the immunosuppressive effect of MDSCs on T cells; this effect could be abolished by IDO inhibitors or STAT3 antagonists.⁸ Then, in study of 2014, we confirmed that breast cancer cell-induced MDSCs upregulated IDO expression via STAT3-stimulated activation of the non-canonical NF- κ B pathway.¹⁹

Chemotherapy can enhance antitumor immunity through a series of different mechanisms. Previous studies found that chemotherapy exerted antitumor effects through cytotoxic effects against tumor cells, in addition to the elimination or inactivation of immunosuppressive cells such as Tregs and MDSCs.^{20,21} McCoy et al. found that patients with low number of stromal Tregs were nearly five times more likely to achieve pCR.²² Romano et al. reported that circulating MDSC subsets were higher in non-responders than responders in patients with Hodgkin lymphoma after chemotherapy.²³ Muller et al. reported that the combination of IDO inhibition and chemotherapy effectively promoted the shrinkage of refractory breast cancers.²⁴ An alternate hypothesis was suggested, whereby fewer suppressive immune cells in the local tumor environment prior to treatment allow effective chemotherapy via inducing antitumor immunity. In this study, we presented an evaluation of Foxp3⁺Treg, CD33⁺MDSC density and IDO expression in pre-treatment diagnostic biopsy samples, with the aim of determining whether pre-NCT infiltrating MDSC density, Treg cell density, IDO expression, and IDO expression in MDSCs were correlated with the subsequent treatment response and survival.

Methods

Patients

Fifty-four women with breast cancer were enrolled in this study. The inclusion criteria of patients were: (1) a diagnosis of advanced breast cancer from the Department of Breast Oncology of Tianjin Medical University Cancer Institute and Hospital between December 1, 2010, and June 30, 2011; (2) histological confirmed breast cancer for all patients and complete information on preoperative immunohistochemistry, including estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (Her-2), p53, and Ki67, were obtained by coarse needle biopsy and postoperative histological information, which were both reviewed based on the 7th AJCC Cancer Staging System; (3) patients were administered different cycles of planned-dose neoadjuvant chemotherapy in accordance with a multi-agent chemotherapy protocol; (4) patients were subjected to mastectomy or lumpectomy in conjunction with either biopsy of ipsilateral sentinel lymph node or axillary resection after the evaluation of the curative effect of NCT and reached R0 resection with a negative surgical margin.

We enrolled 54 patients with a solitary breast tumor in this study; representative formalin-fixed blocks from each coarse needle biopsy samples (at least three tissue cylinders) were selected for immunohistochemical staining. The clinical stages prior to NCT were clinical T1 (3 cases), T2 (20 cases), T3 (20 cases), T4 (11 cases), N0 (13 cases), N1 (16 cases), N2 (18 cases), and N3 (7 cases) and clinical stage II (14 cases), III (38 cases), and IV (2 cases). The pathological stages after surgical resection were pT0 (2 cases), T1 (28 cases), T2 (13 cases), T3 (6 cases), T4 (5 cases) and N0 (9 cases), N1 (16 cases), N2 (13 cases), N3 (16 cases), and stages I (2 cases), II (16 cases), III (34 cases), and IV (2 cases).

The Ethics Committee of Tianjin Cancer Institute and Hospital approved this research project and written consent was obtained from each patient.

Pathologic evaluation

Core biopsy samples and surgical specimens were evaluated in accordance with the Chinese Breast Cancer Guideline. The initial core biopsy samples of the primary tumor were evaluated by standard hematoxylin and eosin (H&E) staining, immunohistochemistry (IHC), and fluorescence or chromogenic in situ hybridization (FISH/CISH) (or both) to determine the histological subtype, the Ki67 index, and the status of ER, PR, and Her-2. The cut-off values for ER, PR, and Ki67 were 10%, 10%, and 20%, respectively.²⁵ Her-2 was scored for intensity and completeness (-, no staining; +, partial weak membranous staining in more than 10% of cancer cells; ++, moderate complete membrane staining in $\geq 10\%$ cancer cells or strong complete membranous staining in $\leq 10\%$ of cancer cells; +++, strong complete membranous staining in $> 10\%$ of cancer cells). Staining at the +++ level was deemed as positive and fluorescence in situ hybridization (FISH) was applied in tumors with ++ staining.²⁶

Immunohistochemistry for IDO, CD33, and Foxp3

Fifty-four tissue samples from breast cancer patients were obtained by coarse needle biopsy prior to NCT, fixed in formaldehyde, and embedded in paraffin wax blocks. Serially cut sections of 4- μ m thickness were generated. The first and last sections from each block were stained with H&E for pathological examination. Others were selected and subjected to immunohistochemical staining in accordance with manufacturer's instruction manuals, as previously reported.²⁷ Each tissue sample was analyzed in 5 sections at least. The tissue sections were incubated with mouse anti-human IDO (Millipore, Billerica, MA), Foxp3 (eBioscience, San Diego, CA), and rabbit anti-human CD33 (Abcam, San Francisco, CA) antibodies (Abs) overnight at 4 °C, and then with goat anti-mouse or anti-rabbit IgG secondary Abs conjugated with streptavidin-HRP (Santa Cruz). The tissue sections were developed by a diaminobenzidine staining kit (Maixin Biotechnology, Fuzhou, China) and observed by an Olympus BX51 microscope (Olympus, Tokyo, Japan). Mouse/rabbit isotype IgG1 was used as the negative controls.

For immunohistochemical double staining, primary Abs against CD33 and IDO stain were performed by the DouSP double-stain kit (Maxim-Bio, Fuzhou City, China), based on the instruction manual. The second Abs were HRP-conjugated anti-mouse IgG and alkaline phosphatase (ALP) conjugated anti-rabbit IgG Abs. IDO⁺ cells were stained red in the cytoplasm by 3-amino-9-ethylcarbazole as substrates for HRP. CD33⁺ cells were stained blue on membrane by 5-bromo-4-chloro-3-indolyl phosphate/NBT as substrate for ALP. The sections were then counterstained with hematoxylin. All sections were observed under an Olympus BX51 microscope.

The levels of positive staining were evaluated by both staining rate (SR) and index (SI). The SR was the percentage of positive cells and defined as an expression score between 0 and 3 (0, <5% of tumor cells were stained; 1, 5–30% were stained; 2, 30–70% were stained; 3, >70% were stained). The SI was assessed based on average of ≥ 5 high-powered fields (400 \times magnification) and scored between 0 and 3 (0, no staining; 1, mild staining; 2, moderate staining; 3, strong staining). The sum of these two parts was designated as the last score. Negative (–) and positive (+) expression was defined as the last score ≤ 3 and last score > 3 , respectively. All samples were reviewed by experienced pathologists who were blinded to the identity of all specimens.

Response to NCT

The clinical response was evaluated by physical examination, mammography, and ultrasonography, based on the Response Evaluation Criteria in Solid Tumors (RECIST 1.1). The clinical complete response (CR) was the disappearance of all known lesions; a clinical partial response (PR) was shrinkage of $\geq 30\%$ in the sum of the longest diameter of the lesion; progressive disease (PD) was defined as a $\geq 20\%$ rise in the sum of the longest diameter of the primary lesion; and stable disease (SD) was defined as insufficient shrinkage to PR or no rise to PD.²⁸

In line with the criteria of the National Surgical Adjuvant Breast and Bowel Project B-18,²⁹ pCR was no invasive carcinoma in the breast at the time of surgery.

Statistical analyses

For categorical variables, the Chi-squared and Fisher's exact tests were used to compare the differences; Spearman's rank correlation test was used to analyze the correlations. Chi-squared and Fisher's exact tests were also applied for the univariate analysis of the factors with significant difference among different responders; the factors that had significant difference in univariate analysis were included in the multivariate logistic regression analysis. The survival curves were analyzed and compared by Kaplan-Meier method and Log-rank test. COX regression was applied to get adjusted hazard ratios for prognosis. $P < 0.05$ were considered statistically significant. All statistical analyses were computed by using SPSS 17.0 software.

Results

The association between tumor-infiltrating CD33⁺MDSCs, Foxp3⁺Tregs, IDO expression, and IDO expression in CD33⁺MDSCs

Foxp3 infiltration staining to quantify Tregs and CD33⁺ staining to quantify infiltrating MDSCs were used because, in our previous study, we demonstrated that the majority ($82.3 \pm 3.1\%$) of CD33⁺ myeloid cells isolated from breast tumor tissues displayed the characteristic phenotype of CD45⁺CD13⁺CD33⁺CD14⁺CD15⁺.⁸ The expression of CD33 (Fig. 1a), IDO (Fig. 1b), Foxp3 (Fig. 1c) in tumor tissues was shown in Fig. 1, in which the negative staining of normal breast tissues was considered to the negative control (NC). In Fig. 1a, CD33 expression was dispersed on membrane of normal cells between cancer cell nests and few in cytoplasm; Fig. 1b showed IDO in cancer tissues, which was mainly expressed in tumor cells and some dispersed in stroma, with brownish yellow staining in cytoplasm; Fig. 1c showed that Foxp3 was expressed in lymphocytes and dispersed in stroma with brownish dark yellow staining in nucleus. The co-expression of IDO and CD33 in tumor tissues, as determined by a double-staining method, was shown in Fig. 1d. The double-positive cells (IDO⁺CD33⁺MDSCs) were red on membrane (CD33) and purple in cytoplasm (IDO).

As shown in Fig. 1e, IDO expression, CD33⁺MDSCs, and Foxp3⁺Tregs were correlated with each other. CD33⁺MDSCs in tumor tissues were positively correlated with IDO expression ($r^2 = 0.418$, $P = 0.002$) and Foxp3⁺Tregs ($r^2 = 0.388$, $P = 0.004$). IDO expression was positively correlated with Foxp3⁺Tregs ($r^2 = 0.487$, $P = 0.000$). Fig. 1f showed that IDO⁺CD33⁺MDSCs were positively correlated with Foxp3⁺Tregs ($r^2 = 0.528$, $P = 0.000$).

The association between tumor-infiltrating CD33⁺MDSC, Foxp3⁺Treg, IDO expression, IDO expression in CD33⁺MDSC, and clinicopathological features

We analyzed the association between tumor-infiltrating CD33⁺MDSCs, Foxp3⁺Tregs, IDO expression, and the clinicopathological features of breast cancer patients.

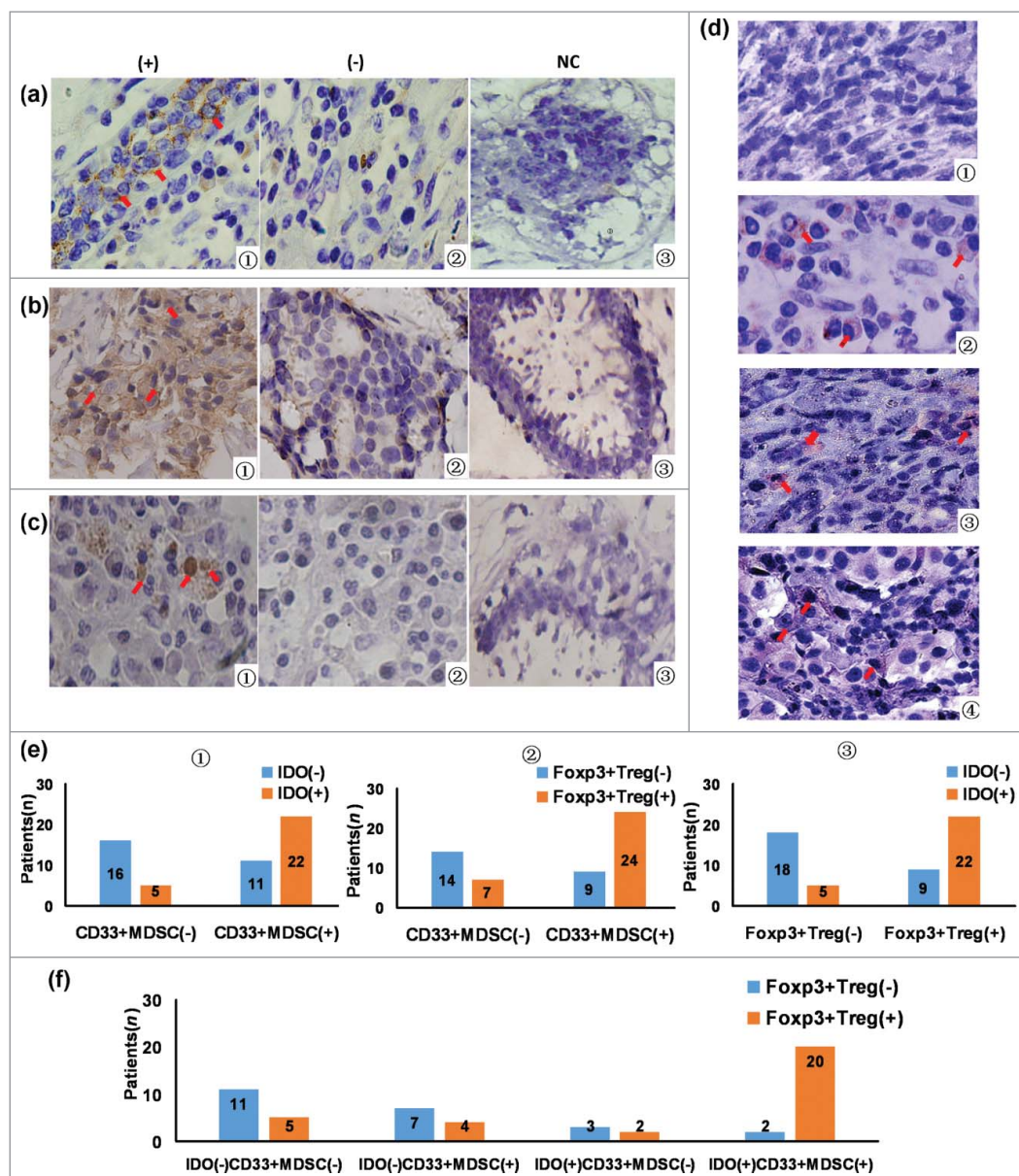


Figure 1. The association between tumor-infiltrating CD33⁺MDSC, Foxp3⁺Treg, IDO expression, and IDO expression in CD33⁺MDSCs. (a1) CD33 in cancer tissues was expressing in normal cells between cancer cell nests dispersed on membrane and few in cytoplasm in cancer tissues ($\times 400$); (a2) negative expression of CD33 in breast cancer tissues ($\times 400$); (a3) negative expression of CD33 in normal breast tissues, which were set as the negative control (NC); (b1) IDO in cancer tissues mainly expressed in tumor cells and some karyocytes dispersed in stroma with brownish yellow staining in cytoplasm ($\times 400$, red arrows); (b2) negative expression of IDO in breast cancer tissues ($\times 400$); (b3) negative control; (c1) in breast cancer tissues, Foxp3 expression in lymphocytes dispersed in stroma with brownish dark yellow staining in nucleus ($\times 400$, red arrows); (c2) negative expression of Foxp3 in breast cancer tissues ($\times 400$); (c3) negative control; (d) co-expression of IDO and CD33 in tumor tissues was determined using immunohistochemical double-staining method; (d1) IDO and CD33 negative staining of breast cancer tissues; (d2) CD33 positive and IDO negative cells (IDO⁻CD33⁺) were stained red on membrane (red arrows); (d3) IDO positive and CD33 negative cells (IDO⁺CD33⁻) were stained purple in cytoplasm (red arrows); (d4) IDO⁺CD33⁺MDSCs were stained red on membrane and purple in cytoplasm ($\times 400$, red arrows); (e1) from the total of 54 patients, 33 patients were positive for CD33⁺MDSCs, 27 patients were positive for IDO expression (IDO positive, orange; negative, blue), and 22 patients were both positive for CD33⁺MDSCs and IDO expression. By Spearman's rank correlation test, it was found that CD33⁺MDSCs were positively correlated with IDO expression ($r^2 = 0.418$, $P = 0.002$); (e2) 31 patients were positive for Foxp3⁺Tregs (Foxp3⁺Treg positive, orange; negative, blue), 33 patients were positive for CD33⁺MDSCs, 24 patients were positive for both CD33⁺MDSCs and Foxp3⁺Tregs. By Spearman's rank correlation test, it was found that CD33⁺MDSCs were positively correlated with Foxp3⁺Tregs ($r^2 = 0.388$, $P = 0.004$); (e3) 31 patients were positive for Foxp3⁺Tregs, 27 patients were positive for IDO expression, 22 patients were positive for both Foxp3⁺Treg and IDO expression (IDO positive, orange; negative, blue). By Spearman's rank correlation test, it was found that IDO expression was positively correlated with Foxp3⁺Tregs ($r^2 = 0.487$, $P = 0.000$); (f) 31 patients were positive for Foxp3⁺Tregs, 22 patients were positive for IDO expression in CD33⁺MDSCs, 20 patients were positive for both Foxp3⁺Tregs and IDO expression in CD33⁺MDSCs. By Spearman's rank correlation test, IDO⁺CD33⁺MDSCs were positively correlated with Foxp3⁺Tregs ($r^2 = 0.528$, $P = 0.000$).

CD33⁺MDSCs ($P = 0.000$), Foxp3⁺Tregs ($P = 0.001$), and IDO expression ($P = 0.005$) were associated with advanced clinical T stage. The positivity of IDO expression and Foxp3⁺Treg were more frequently expressed in patients with breast cancer at the N1–N3 stage ($P = 0.051$ and $P = 0.054$), as shown in Table 1.

We analyzed IDO/CD33 co-expression to confirm the association between IDO expression in CD33⁺MDSCs and the clinicopathological features prior to NCT. The IDO⁺ expression in CD33⁺MDSCs was correlated with advanced clinical T stage ($P = 0.000$) and ER(+) ($P = 0.031$, Table 2).

Table 1. The association between tumor-infiltrating CD33⁺MDSC, Foxp3⁺Treg, IDO expression and clinicopathological features prior NCT.

Clinicopathological features	CD33 ⁺ MDSC				Foxp3 ⁺ Treg				IDO				
	-	+	χ^2	P	-	+	χ^2	P	-	+	χ^2	P	
<i>n</i>	21	33			23	31			27	37			
Age	<50	8	18	1.391	0.275	12	14	0.260	0.784	15	11	1.187	0.414
	≥50	13	15			11	17			12	16		
Menopausal status	Pre-	13	15	1.391	0.275	11	17	0.260	0.784	13	15	0.297	0.786
	Post-	8	18			12	14			14	12		
History of gravidity	No	4	3	1.128	0.411	5	2	2.735	0.122	6	1	4.103	1.000
	Yes	17	30			18	29			21	26		
Family history of malignance	No	14	26	0.982	0.355	19	21	1.520	0.347	21	19	0.386	0.757
	Yes	7	7			4	10			6	8		
Clinical T stage	T1, T2	16	7	15.864	0.000	16	7	11.921	0.001	17	6	9.164	0.005
	T3, T4	5	26			7	24			10	21		
Clinical N stage	N0	7	6	1.612	0.328	9	4	4.969	0.051	10	3	4.964	0.054
	N1-N3	14	27			14	27			17	24		
Clinical stage	I, II	6	8	0.125	0.758	8	6	1.636	0.226	9	5	1.543	0.352
	III, IV	15	25			15	25			18	22		
Histological type	IDBC	17	27	0.003	1.000	22	22	5.332	0.032	23	21	0.491	0.728
	Other	4	6			1	9			4	6		
ER	-	9	15	0.035	1.000	11	13	0.186	0.784	16	8	4.800	0.054
	+	12	18			12	18			11	19		
PR	-	13	14	1.948	0.264	11	16	0.076	1.000	14	13	0.074	1.000
	+	8	19			12	15			13	14		
Her-2	-	15	18	1.539	0.261	16	17	1.205	0.398	19	14	1.948	0.246
	+	6	15			7	14			8	13		
Ki67	-	10	12	0.673	0.571	13	9	4.133	0.054	14	8	2.761	0.166
	+	11	21			10	22			13	19		
P53	-	7	16	1.205	0.398	8	15	0.999	0.407	13	10	0.682	0.583
	+	14	17			15	16			14	17		
Luminal type	Luminal	16	26	0.751	0.687	18	24	0.176	0.916	19	23	4.381	0.112
	Her-2	1	3			2	2			4	0		
	Basal-like	4	4			3	5			4	4		

IDBC: Invasive ductal breast cancer.

Table 2. The association between co-expression of IDO/CD33 and clinicopathological features prior NCT.

Clinicopathological features	<i>n</i>	IDO ⁻ CD33 ⁻	IDO ⁻ CD33 ⁺	IDO ⁺ CD33 ⁻	IDO ⁺ CD33 ⁺	χ^2	P	
Total	54	16	11	5	22			
Age	<50	26	7	8	1	10	4.437	0.218
	≥50	28	9	3	4	12		
Menopausal status	Pre-	28	9	4	4	11	2.798	0.424
	Post-	26	7	7	2	10		
History of gravidity	No	7	3	3	1	0	5.967	0.133
	Yes	47	13	8	4	22		
Family history of malignant	No	40	11	10	3	16	2.396	0.494
	Yes	14	5	1	2	6		
Clinical T stage	T1, T2	23	14	3	2	4	19.627	0.000
	T3, T4	31	2	8	3	18		
Clinical N stage	N0	13	6	4	1	2	5.234	0.155
	N1-N3	41	10	7	4	20		
Clinical stage	I, II	14	4	5	2	3	4.430	0.218
	III, IV	40	12	6	3	19		
Histological type	IDBC	44	13	10	4	17	0.914	0.822
	Other	10	3	1	1	5		
ER	-	24	7	9	2	6	8.893	0.031
	+	30	9	2	3	16		
PR	-	27	10	4	3	10	2.200	0.532
	+	27	6	7	2	12		
Her-2	-	33	11	8	4	10	4.037	0.122
	+	21	5	3	1	12		
Ki67	-	22	8	6	2	6	3.090	0.378
	+	32	8	5	3	16		
P53	-	23	6	7	1	9	3.231	0.357
	+	21	10	4	4	13		
Luminal type	Luminal	42	12	7	4	19	8.921	0.178
	Her-2	4	1	3	0	0		
	Basal-like	8	3	1	1	3		

IDBC: Invasive ductal breast cancer.

Table 3. Univariate analysis of clinicopathological features with respect to breast cancer patients' response to NCT.

Clinicopathological features		Total	Clinical response				Pathological response			
			CR+PR(n)	PD+SD(n)	χ^2	P	pCR (n)	No-pCR (n)	χ^2	P
Age	<50	26	13	13	2.605	0.183	5	21	0.017	1.000
	≥50	28	20	8			5	23		
Menopausal status	Pre-	28	20	8	2.605	0.183	5	23	0.017	1.000
	Post-	26	13	13			5	21		
History of gravidity	No	7	5	2	0.360	0.693	2	5	0.539	0.601
	Yes	47	28	19			8	39		
Family history of malignance	No	40	24	16	0.080	1.000	6	34	1.266	0.424
	Yes	14	9	5			4	10		
Clinical T stage	T1, T2	23	18	5	4.958	0.047	8	15	7.024	0.012
	T3, T4	31	15	16			2	29		
Clinical N stage	N0	13	8	5	0.001	1.000	5	8	4.513	0.048
	N1-N3	41	25	16			5	36		
Clinical stage	I+II	14	8	6	0.125	0.758	3	11	0.106	0.708
	III+IV	40	25	15			7	33		
Pathological T stage	pT0, T1, T2	43	30	13	6.656	0.015	8	34	0.814	0.667
	pT3, T4	11	3	8			2	10		
Pathological N stage	pN0	9	5	4	0.140	0.708	0	9	2.455	0.183
	pN1-N3	45	28	17			10	35		
Pathological TNM stage	I+II	18	10	8	0.351	0.569	3	15	0.061	1.000
	III+IV	36	23	13			7	29		
Histological type	IDBC	44	27	17	0.006	0.936	7	37	1.072	0.370
	Other	10	6	4			3	7		
ER	-	24	19	5	5.926	0.015	5	19	0.153	0.736
	+	30	14	16			5	25		
PR	-	27	20	7	3.818	0.093	6	21	0.491	0.728
	+	27	13	14			4	23		
Her-2	-	33	18	15	1.539	0.261	9	24	4.310	0.069
	+	21	15	6			1	20		
Ki67	-	22	15	7	0.781	0.410	5	17	0.436	0.723
	+	32	18	14			5	27		
P53	-	23	13	10	0.355	0.584	3	20	0.796	0.489
	+	21	20	11			7	24		
Luminal type	Luminal	42	22	20	6.237	0.044	6	36	2.520	0.284
	Her-2	4	4	0			1	3		
	Basal-like	8	7	1			3	5		
Chemotherapy regime	TAC/TEC	39	26	13	1.834	0.100	6	33	1.747	0.418
	TA/TE	13	6	7			3	10		
	Other	2	1	1			1	1		
Chemotherapy cycles	2 cycles	39	27	12	3.895	0.065	5	34	3.021	0.119
	≥3 cycles	15	6	9			5	10		

IDBC: Invasive ductal breast cancer; For categorical variables, the χ^2 value and P value were calculated by Chi-square tests and Fisher's exact tests.

The association between tumor-infiltrating CD33⁺MDSC, Foxp3⁺Treg, IDO expression, IDO expression in CD33⁺MDSC, and response to NCT

In Table 3 and, the univariate analysis of clinicopathological features with respect to the patients' responses to NCT was shown, whereas Table 4 showed the univariate analysis of

immunological features with respect to the patients' responses to NCT.

Positive results for tumor infiltrating Foxp3⁺Tregs ($P = 0.047$), CD33⁺MDSCs ($P = 0.023$), IDO expression ($P = 0.024$), IDO expression in CD33⁺MDSCs ($P = 0.014$) and clinical T3-T4 stage ($P = 0.047$), pathological T3-T4 stage ($P = 0.015$), ER positivity ($P = 0.015$), and luminal type or

Table 4. Univariate analysis of immunological features with respect to breast cancer patients' response to NCT.

Clinicopathological features		Total	Clinical response				Pathological response			
			CR+PR(n)	PD+SD(n)	χ^2	P	pCR (n)	No-pCR (n)	χ^2	P
Foxp3 ⁺ Treg	-	23	18	5	4.958	0.047	7	16	3.770	0.078
	+	31	15	16			3	28		
CD33 ⁺ MDSC	-	21	17	4	5.692	0.023	7	14	4.998	0.035
	+	33	16	17			3	30		
IDO	-	27	21	6	6.312	0.024	7	20	1.964	0.293
	+	37	12	15			3	24		
IDO ⁺ CD33 ⁺ MDSC	IDO ⁻ /CD33 ⁻	16	15	1	10.578	0.014	5	11	6.095	0.107
	IDO ⁻ /CD33 ⁺	11	6	5			2	9		
	IDO ⁺ /CD33 ⁻	5	2	3			2	3		
	IDO ⁺ /CD33 ⁺	22	10	12			1	21		

Table 5. Multivariate analysis of immunological and clinicopathological features with respect to breast cancer patients' clinical response to NCT.

Features		β	SE	Wald	df	P	OR(95% CI)
CD33 ⁺ MDSC	–						1
	+	4.040	1.501	7.246	1	0.007	56.843(3.000~1077.076)
IDO	–						1
	+	3.286	1.518	4.687	1	0.030	26.743(1.365~523.973)
IDO ⁺ CD33 ⁺ MDSC	Other						1
	IDO ⁺ CD33 ⁺	4.981	2.010	6.139	1	0.013	145.553(2.831~7483.474)
Pathological T stage	pT0, T1, T2						1
	pT3, T4	2.659	1.020	6.791	1	0.009	14.287(1.933~105.574)
ER	–						1
	+	1.705	0.968	3.100	1	0.078	5.502(0.825~36.709)

β : regression coefficient; SE: standard error; Wald: wald chi-square; df: degree of freedom; OR: odds ratio.

Her-2 and Basal-like ($P = 0.044$) were correlated with PD+SD in univariate analysis. In this study, $P < 0.05$ was considered to be statistically significant; however, the statistical significance of Foxp3⁺Treg ($P = 0.044$), clinical T stage ($P = 0.047$), and luminal type ($P = 0.047$) were borderline. Therefore, we excluded these three factors in the multivariate analysis. Finally, we included CD33⁺MDSCs, IDO expression, IDO expression in CD33⁺MDSCs, pathological stage, and ER(+) in multivariate logistic regression analysis. These results showed that CD33⁺MDSCs [OR: 56.843(3.000–1077.076), $P = 0.007$], IDO expression [OR: 26.743(1.365–523.973), $P = 0.030$], IDO expression in CD33⁺MDSCs [OR: 145.553(2.831–7483.474), $P = 0.013$], and advanced pathological T stage [OR: 14.287(1.933–105.574), $P = 0.009$] were risk factors for PD+SD (Table 5).

When we concentrated on the pCR of NCT, only the positive expression of CD33⁺MDSCs ($P = 0.035$), clinical T3–T4 stage ($P = 0.012$), and clinical N1–N3 stage ($P = 0.048$) were associated with no-pCR in the univariate analyses (Table 3 and Table 4). These significant features were then included in the multivariate logistic regression analysis, which showed that advanced clinical T stage [OR: 10.322(1.109–96.078), $P = 0.040$] and clinical N stage [OR: 7.366(1.111–48.852), $P = 0.039$] were significant risk factors for no-pCR (Table 6).

Prognostic analysis

In the survival analysis, clinical stage prior NCT was significantly correlated with PFS ($P = 0.021$, Fig. 2a), clinical T3–T4 stage (Fig. 2b) and Foxp3⁺Treg (Fig. 2g and Fig. 2h) were significantly correlated with shorter overall survival (OS, $P = 0.001$ and $P = 0.022$, respectively). However, patients positive for CD33⁺MDSCs (Fig. 2c and Fig. 2d), IDO expression (Fig. 2e and Fig. 2f), and IDO expression in CD33⁺MDSCs (Fig. 2i

and Fig. 2j) inclined to poorer prognosis. The 1, 3 and 5 year survival rates of breast cancer patients according to all immunological features, clinicopathological features and therapies were shown in supplementary Table 1. In Table 7, we used COX regression to adjust covariates, including Clinical T stage, Clinical N stage, Clinical stage, Pathological T stage, Pathological N stage, Pathological stage, Histological type, ER status, PR status, Her-2, Luminal type, Clinical response, pathological response, surgery method, post-operative chemotherapy, post-operative radiation therapy, post-operative hormone therapy, to get adjusted hazard ratios for prognosis. Foxp3⁺Treg was significant risk factors for overall survival [HR: 34107.438(17.192~664331.905), $P = 0.007$].

Discussion

MDSCs and Tregs play a key role in immunosuppressive networks and contribute to tumor immune toleration. Owing to the multiple factors and the multiple steps of immunoregulation that contribute to the complicate immunoregulatory mechanisms in the development of cancer, each patient had different immune characteristics based on different pathologies, including tumor progression, stage, and basic disease. However, previous studies had demonstrated that elevated MDSCs and Tregs were associated with advanced disease stage and poor prognosis in various malignancies.^{4-6,30,31} Both MDSCs and Tregs were also confirmed to be correlated with chemotherapy outcomes in various malignant cancers.²⁰

Mizukoshi et al. found that MDSCs prior to treatment were significantly lower in patients who achieved CR and PR by hepatic arterial infusion chemotherapy than in those SD or PD.³² MDSCs <30.5% was an important prognostic factor for longer survival after hepatic arterial infusion chemotherapy. In the study of Kawano et al., the G-CSF-induced increase of MDSCs in situ contributed to the chemoresistance of cervical

Table 6. Multivariate analysis of immunological and clinicopathological features with respect to breast cancer patients' pathological response to NCT.

Features		β	SE	Walds	df	P	OR(95% CI)
CD33 ⁺ MDSC	–						1
	+	0.260	0.960	0.073	1	0.787	1.297(0.198~8.505)
Clinical T stage	pT0, T1, T2						1
	T3, T4	2.334	1.138	4.206	1	0.040	10.322(1.109~96.078)
Clinical N stage	N0						1
	N1-N3	1.997	0.965	4.279	1	0.039	7.366(1.111~48.852)

β : regression coefficient; SE: standard error; Wald: wald chi-square; df: degree of freedom; OR: odds ratio.

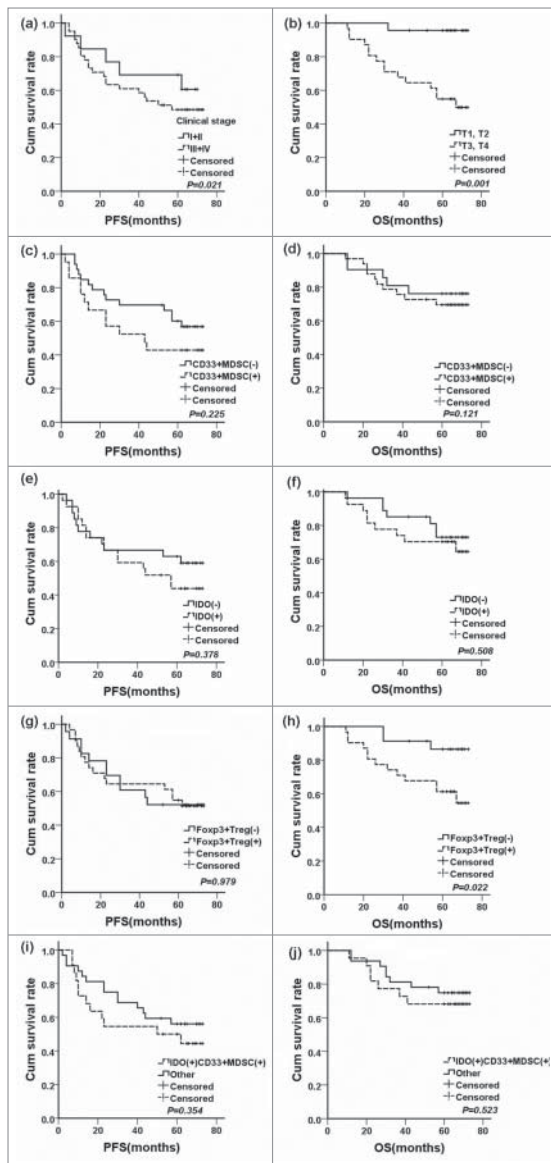


Figure 2. The survival curves for breast cancer patients treated by NCT. (a) PFS curves arranged by clinical stage ($P = 0.021$); (b) OS curves arranged by clinical T stage ($P = 0.001$); (c) PFS curves arranged by tumor-infiltrating CD33⁺MDSCs ($P = 0.225$); (d) OS curves arranged by tumor-infiltrating CD33⁺MDSCs ($P = 0.121$); (e) PFS curves arranged by IDO expression ($P = 0.378$); (f) OS curves arranged by IDO expression ($P = 0.508$); (g) PFS curves arranged by tumor-infiltrating Foxp3⁺Tregs ($P = 0.979$); (h) OS curves arranged by tumor-infiltrating Foxp3⁺Tregs ($P = 0.022$); (i) PFS curves arranged by IDO expression in CD33⁺MDSCs ($P = 0.354$); (j) OS curves arranged by IDO expression in CD33⁺MDSCs ($P = 0.523$).

cancer with G-CSF producing tumors,³³ and the elimination of MDSCs sensitized cervical cancers to cisplatin. Romano et al. showed that all MDSC subsets were elevated in patients with Hodgkin lymphoma compared with healthy controls and were higher in non-responders treated upfront.²³ In our study, tumor-infiltrating CD33⁺MDSCs were lower in patients who achieved CR+PR and pCR, lower CD33⁺MDSCs was also related to longer PFS and OS.

In a recent study that focused on Tregs, McCoy et al. found that tumor-infiltrating Tregs prior to NCT for rectal tumors were not correlated with response.³⁴ However, in their previous study in 2016, a low density of Foxp3⁺ Tregs within the tumor stroma after NCT was shown to be strongly associated with

pCR.²² Baras et al. reported that the ratio of CD8⁺T cells to Tregs in situ was significantly related to a response to NCT in patients with muscle invasive urothelial cancer of the bladder.³⁵ Roselli et al. showed the frequency of Tregs prior to therapy associated with PFS. The Treg of responders was reduced more obviously after first-line FOLFIRI plus bevacizumab therapy in comparison with non-responders.³⁶ Kotsakis et al. found that CD4⁺Tregs were increased in NSCLC and correlated with the response and prognosis; patients at stage IV who achieved PD in chemotherapy had a significantly elevated initial naive Treg ratio.¹¹ Kim et al. found that, in vivo and vitro, the combination of cisplatin and inhibiting Treg cell migration strengthened the anti-cancer outcome in comparison with a single treatment.³⁷ In our study, Foxp3⁺Tregs were also associated with CR+PR in univariate analysis, which was in agreement with the studies mentioned above. However, in multivariate logistic regression analysis, this association was not confirmed. In prognostic analysis, the Foxp3⁺Tregs were significantly correlated with a shorter OS.

Limagne et al. reported that both Treg and MDSC accumulation in metastatic colorectal cancer were also correlated with a poor outcome.⁷ Previous reports have suggested that MDSCs may contribute to Treg induction in cancer.³⁸ In a study of human fibrocytic MDSCs, Zoso et al. found that MDSCs that expressed IDO and enhanced immune suppression by Treg cell expansion.¹⁵ IDO is an important mechanism by which effector T cells can be induced and converted to Tregs. The IDO-mediated depletion of tryptophan and subsequent production of immunosuppressive products such as kynurenine may also lead to T cell suppression through the down-regulation of the TCR-CD3- ζ chain.^{13,14,16} So, IDO exerts its suppressive effects via blocking the initiation of Ag-specific immune response and antitumor cytotoxicity of activated T cells, and to increase Tregs.^{17,18} In our previous study, we also found that IDO expression was significantly up-regulated in MDSCs and this higher expression was associated with increased Foxp3⁺Tregs. Breast cancer-induced MDSCs can up-regulate IDO expression via STAT3-stimulated activation of the non-canonical NF- κ B pathway.¹⁹ The inhibition of IDO by 1-MT can significantly attenuate MDSC-regulated immunosuppression of T cell proliferation and Th1 polarization, which suggests that the immunosuppressive activity of MDSCs was IDO-dependent.⁸ In the cancers that highly express IDO, MDSCs are an important cell group in promoting cancer growth and immunotherapy resistance.³⁹ In this study, we found that CD33⁺MDSCs and IDO expression were positively correlated with Foxp3⁺Treg, respectively. IDO expression in CD33⁺MDSCs showed a significant positive correlation with Foxp3⁺Tregs. When focused on the clinical aspects, higher IDO expression in MDSCs were correlated with advanced clinical T stage and ER(+), poorer survival, and more frequent achievement of patients in PD+SD.

These findings had many limitations: first, this was a relatively small patient cohort from a single center; thus, these results must be confirmed in larger sample and multi-center studies; second, we applied Foxp3 for labeling Treg, which is commonly used, and used CD33 staining to quantify infiltrating MDSCs based on our previous study. However, we also recognized that Foxp3 and CD33 could also found in activated CD4⁺T cells and other subgroups of myeloid cells in humans.

Table 7. COX regression for prognostic significance of immunological features for breast cancer patients.

Features	β	SE	Wald	df	P	HR(95% CI)		
PFS	Foxp3 ⁺ Treg	–				1		
		+	–0.156	0.887	0.031	1	0.861	0.856(0.151~4.866)
	CD33 ⁺ MDSC	–				1		
		+	2.826	1.506	3.523	1	0.061	16.873(0.882~322.662)
	IDO	–				1		
OS		+	0.626	1.254	0.250	1	0.617	1.871(0.160~21.841)
	IDO ⁺ CD33 ⁺ MDSC	Other				1		
		IDO ⁺ CD33 ⁺	–1.963	1.846	1.131	1	0.288	0.140(0.004~5.236)
	Foxp3 ⁺ Treg	+	10.437	3.874	7.259	1	0.007	34107.438(17.192~664331.905)
	CD33 ⁺ MDSC	–				1		
OS		+	–0.448	1.349	.110	1	0.740	0.639(0.045~8.988)
	IDO	–				1		
		+	6.788	4.342	2.444	1	0.118	887.195(0.179~67700.393)
	IDO ⁺ CD33 ⁺ MDSC	Other				1		
		IDO ⁺ CD33 ⁺	1.689	3.104	.296	1	0.586	5.412(0.012~2374.547)

β : regression coefficient; SE: standard error; Wald: wald chi-square; df: degree of freedom; HR: hazard ratio.

To get adjusted hazard ratios, COX regression was applied for adjusting covariates, which included Clinical T stage, Clinical N stage, Clinical stage, Pathological T stage, Pathological N stage, Pathological stage, Histological type, ER status, PR status, Her-2, Luminal type, Clinical response, Pathological response, Surgery method, Post-operative chemotherapy, Post-operative radiation therapy, Post-operative hormone therapy.

Additionally, these data are only correlative analyses and further experimental study is required to explore the underlying mechanisms of these correlations.

Based on our study, these positive immunosuppressive features in preoperative evaluation may be suggestive for the need of more aggressive surgical resection and chemotherapy to achieve better therapeutic outcome and survival in cancer treatment. Recently, the inhibition of IDO was applied in a phase II-II clinical trial,^{24,39} and the immunotherapy target to key checkpoints which also inhibit Tregs and MDSCs had also been entered into clinical trials.⁴⁰ Therefore, the combination of immunotherapy and chemotherapy may improve the clinical efficacy of chemotherapy. In particular, for patients with these immunosuppressive features, the use of targeted immunotherapy as an adjuvant therapy may be an effective choice to prolong the survival time.

In conclusion, this study proved that tumor-infiltrating Tregs, MDSCs, and IDO expression, and IDO expression in MDSCs were correlated with each other and with clinicopathological features. The tumor-infiltrating Treg was a significant factor for poor OS. These findings suggested that tumor-infiltrating Tregs, MDSCs, and IDO expression were predictable markers for the outcome of NCT and might help clinical decision-making for the delivery of improved therapies for breast cancer.

Declarations

- Ethics approval and consent to participate: This research project was approved by the Ethics Committee of Tianjin Cancer Institute and Hospital. Written consents were obtained from each patient.
- Consent to publish: Consent to publish from the participant to report individual patient data were obtained from each patient.
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Zhao and Shixia Li was analyzed and interpreted and revised the manuscript. All authors read and approved the final manuscript.

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