



M2 tumor-associated macrophages play important role in predicting response to neoadjuvant chemotherapy in triple-negative breast carcinoma

Vidya Arole¹ · Hiroaki Nitta² · Lai Wei³ · Tiansheng Shen¹ · Anil V. Parwani¹ · Zaibo Li¹

Received: 16 April 2021 / Accepted: 11 May 2021 / Published online: 25 May 2021

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Abstract

Purpose Two types of macrophages are present in tumor microenvironment. M1 macrophages exhibit potent anti-tumor properties, while M2 macrophages play the pro-tumoral roles. The presence of M2 macrophages is associated with worsened overall survival in triple-negative breast carcinoma (TNBC) patients. However, the relationship between M2 macrophages and response to neoadjuvant chemotherapy (NAC) is unknown.

Methods M2 macrophages were investigated on biopsy whole sections from 66 TNBCs treated with NAC by CD163 together with other immune checkpoint markers (PD1, PD-L1 and CD8) using a multi-color immunohistochemical multiplex assay.

Results Incomplete response was significantly associated with older age, lower PD-L1 expression (tumor and stroma), lower levels of CD8-positive TILs in stroma, but higher level of CD163-positive macrophages, with the level of CD163-positive M2 macrophages in peritumoral area as the strongest factor.

Conclusions Our data have demonstrated that the level of CD163-positive M2 macrophages was significantly higher in TNBC patients with incomplete response than patients with complete response, suggesting M2 macrophages' important role in predicting TNBC patients' response to NAC.

Keywords M2 macrophage · PD-L1 · Cytotoxic T cells · Tumor-associated lymphocytes · Triple-negative breast carcinoma · Neoadjuvant therapy · Pathologic complete response

Introduction

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype, which is estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative [1]. Neoadjuvant chemotherapy (NAC) has been increasingly given to TNBC patients with improved long-term outcomes in TNBC patients [2, 3]. Response to NAC is commonly evaluated by microscopically examining surgically

resected specimen and pathologic complete response (pCR) is defined as no residual invasive carcinoma.

The presence of tumor-infiltrating lymphocytes (TILs) in tumor microenvironment is associated better response to NAC in breast cancer patients including TNBC patients, suggesting that the immune defense system may augment NAC-induced tumor cell death [4–8]. Additionally, the expression of programmed cell death 1 ligand (PD-L1) has been demonstrated to be associated with response to immunotherapy and/or chemotherapy and overall survival in breast cancer patients, especially TNBC patients. [9–13].

Tumor microenvironment also contains tumor-associated macrophages (TAMs), which play important roles in tumor progression [14–17]. There are two types of TAMs: M1 macrophages are pro-inflammatory and suppress tumor cells, while M2 macrophages are immunosuppressive and promote tumor growth [14, 16]. M2 macrophages are related to hormonal status, stage, lymph node status, and poor survival in breast cancers [18–23].

✉ Zaibo Li
Zaibo.Li@osumc.edu

¹ Department of Pathology, Wexner Medical Center, The Ohio State University, 410 W. 10th Ave, Columbus, OH 43210, USA

² Roche Tissue Diagnostics, Tucson, AZ 85755, USA

³ Department of Biomedical Informatics, Center for Biostatistics, The Ohio State University, Columbus, OH 43210, USA

To understand the relationship between M2 macrophages and response to NAC, we undertook an evaluation of M2 macrophages together with other immune markers (CD8+cytotoxic T cells and PD-L1) expression in TNBCs and its association with pCR after NAC.

Patients and methods

Patients, specimens and pathological assessment of the response to neoadjuvant therapy

This study was approved by The Ohio State University institutional research board. Informed consent was obtained from all individual patients included in the study. Sixty-six triple-negative invasive breast carcinoma patients treated with neoadjuvant chemotherapy and follow-up surgical resection (39 lumpectomy specimens and 27 mastectomy specimens) were included in this study. For neoadjuvant chemotherapy, all patients received four cycles of AC (doxorubicin+cyclophosphamide) together with Taxol (paclitaxel or docetaxel).

Surgical resection specimens were carefully examined grossly and microscopically to search any residual tumor. When residual invasive tumor was not detected in breast tissue and metastasis was not detected in lymph node, pCR was rendered. The entire tumor bed(s) was submitted for histological examination for cases with pCR.

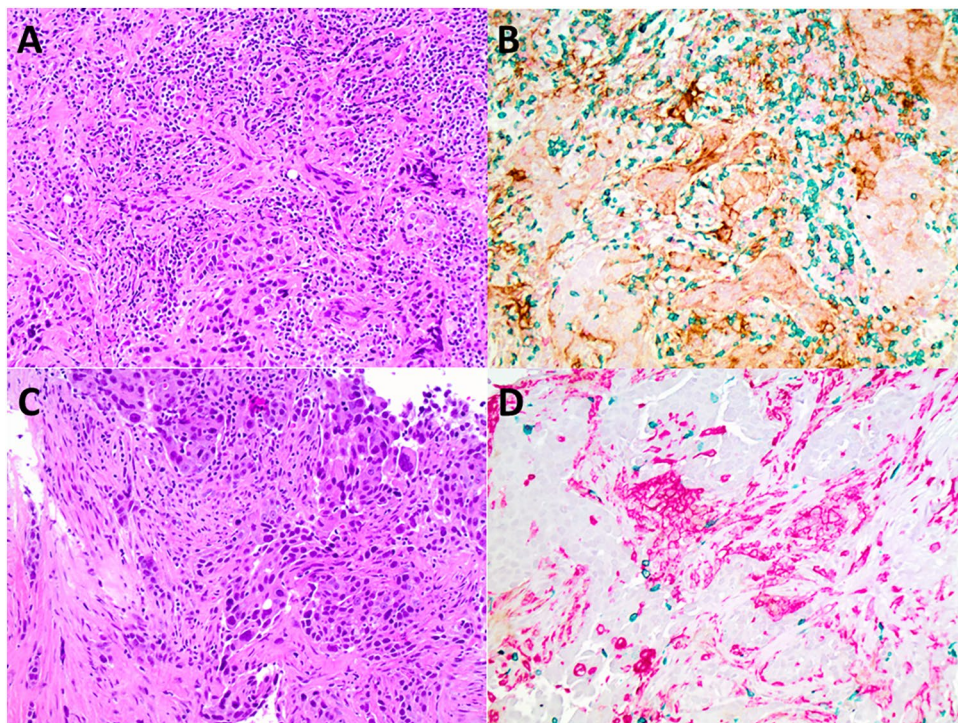
Evaluation of M2 macrophage, cytotoxic T cells and PD-L1 expression using multi-color multiplex immunohistochemistry with CD163, CD8 and PD-L1

M2 macrophages are usually characterized by CD163 expression [21, 24]. We evaluated M2 macrophages, cytotoxic T cells and PL-L1 expression by applying a multi-color multiplex immunohistochemistry (IHC) with three distinct antibodies including CD163 (clone SP57, rabbit; Ventana Medical Systems, Inc), CD8 (clone MRQ26, mouse; Ventana Medical Systems, Inc,) and PD-L1 (clone SP263, rabbit; Ventana Medical Systems, Inc, Tucson, AZ, USA). IHCs were performed on freshly cut whole sections from pre-NAC biopsy specimens. M2 macrophages and cytotoxic T cells were evaluated by estimating the percentage of tumoral or stromal areas infiltrated by CD163-positive cells and CD8-positive cells, respectively. PD-L1 expression was evaluated by estimating the percentage of tumor cells or stromal (immune) cells with specific membranous PD-L1 staining. Representative images with different combinations of CD163 (red), CD8 (green) and PD-L1 (brown) expression are illustrated in Fig. 1.

Statistical analyses

Statistical analysis was performed using SAS version 9.4 for Windows (SAS Institute, Inc, Cary, NC, USA). Descriptive statistics were used to summarize patient clinical and

Fig. 1 Representative images of different immune reaction and PD-L1 expressions in triple-negative breast carcinoma, as detected with anti-PD-L1 multiplex immunohistochemistry (anti-CD8 in green, anti-CD163 in red, and anti-PD-L1 in brown). **A, B** One pCR case with high PD-L1, high CD8, but low CD163; **C, D** One incomplete case with no PD-L1, low CD8, but high CD163. **A, C:** H&E stains, **B, D:** multiplex immunohistochemistry, ($\times 200$)



pathologic characteristics. Categorical data were summarized as frequency and percentage, and continuous variables as medians and ranges. To study the associations with pCR, Fisher's exact was used for categorical variables. Wilcoxon rank-sum test was used to compare the continuous variables. All the continuous variables have been tested for normality using Kolmogorov–Smirnov test. A multivariable logistic regression model was used to determine the variables associated with the incidence of death as well as recurrence. Variables with a p value < 0.10 in the univariate analysis were entered into a multivariable model. Variables were removed sequentially from the multivariable model using the backward selection method.

Results

Clinicopathologic characteristics of 66 triple-negative breast carcinomas with neoadjuvant chemotherapy and subsequent resection

The clinical and pathologic findings are summarized in Table 1. The median age at diagnosis was 51 years (26–74 years). Sixty-four cases were invasive ductal carcinoma NOS, and the other two cases were metaplastic carcinoma. All cases were Nottingham grade 3 or 2 with an average of 2.85. Fifty-nine cases had negative HER2 IHC, and the other seven cases had equivocal HER2 IHC, but negative HER2 fluorescence in-situ hybridization (FISH). Twenty-eight cases (42.4%) had pCR. Among 38 cases with residual tumor, 16 had lymph nodal metastasis.

Univariate analysis of factors associated with response to NAC in 66 triple-negative breast carcinomas

Twenty-eight cases (42.4%) had complete response (pCR) and 38 (57.6%) had residual tumor. Among 66 cases, PD-L1 expression in tumor cells was identified in 32 (48.5%) cases with at least 1%. PD-L1 expression in stromal immune cells was identified in 35 (53%) cases. CD8-positive T cell infiltrate was identified in tumoral area for 56 (87.9%) cases and in peritumoral stromal area for 64 (97.0%) cases. CD163-positive M2 macrophages were found in tumoral area for 65 (98.5%) cases and in peritumoral area for all cases.

Univariate analysis was performed to examine the associations between the response to NAC and clinicopathologic and immune reaction variables, including age, grades, HER2 IHC, PD-L1, CD8 and CD163. The complete response group showed significantly younger age (47 vs 53.2, $p = 0.041$); increased PD-L1 expression in both tumor cells and stromal immune cells (tumoral: 7.8% vs 2.6%, $p = 0.004$; stromal: 7.3% vs 1.9%, $p = 0.007$);

Table 1 Demographic characteristics of study cohort with 66 triple-negative breast carcinomas

	#/Median (average)	%/Range
Total case	66	
Age	51	26–74
Histologic type		
Ductal	64	97.0%
Metaplastic	2	3.0%
Nottingham grade	2.85	2–3
Tubule	2.98	2–3
Nuclear	2.86	2–3
Mitosis	2.32	1–3
HER2		
0–1 +	59	89.4%
2 +	7	10.6%
Neoadjuvant chemotherapy		
Complete response	28	42.4%
Residual tumor	38	57.6%
T stage		
ypT0	28	42.4%
ypT1	31	47.0%
ypT2	7	10.6%
N stage		
ypNx	6	9.1%
ypN0	44	66.7%
ypN1	11	16.7%
ypN2	5	7.6%

increased CD8-positive cells in both tumoral and stromal areas (tumoral: 11% vs 7.4%, $p = 0.004$; stromal: 15% vs 8.7%, $p = 0.001$); but decreased CD163-positive M2 macrophages in both tumoral and stromal areas (tumoral: 22.8% vs 35.9%, $p = 0.003$; stromal: 24.8% vs 39.2%, $p = 0.003$) (Table 2).

Multivariable analysis of factors associated with response to NAC in 66 triple-negative breast carcinomas

A multivariable logistic regression model was then performed to analyze the variables with $p < 0.10$ in the univariate analysis (age, mitosis, PD-L1, CD8 and CD163). Variables with $p > 0.05$ were removed sequentially from the multivariate model using backward selection method. In this model only increased mitosis ($p = 0.031$), increased PD-L1 expression in stromal immune cells ($p = 0.005$) and decreased CD163-positive M2 macrophages in stromal area ($p < 0.001$) were significantly associated with complete response to NAC (Table 3).

Table 2 Univariate analysis of factors associated with response to neoadjuvant chemotherapy in 66 triple-negative breast carcinomas

Variable	Level	Incomplete response (n=38)	Complete response (n=28)	p value
Age	Mean (min, max)	53.2 (31, 74)	47 (26, 72.9)	0.041
Histology type	Ductal	36 (95%)	28 (100%)	0.504
	Metaplastic	2 (5%)	0 (0%)	
Nottingham grade	2	7 (18%)	3 (11%)	0.498
	3	31 (82%)	25 (89%)	
Tubule	2	1 (3%)	0 (0%)	1
	3	37 (97%)	28 (100%)	
Nuclear	2	6 (16%)	3 (11%)	0.722
	3	32 (84%)	25 (89%)	
Mitosis	1	3 (8%)	1 (4%)	0.078
	2	25 (66%)	12 (43%)	
	3	10 (26%)	15 (54%)	
HER2 IHC score	0/1 +	34 (89%)	25 (89%)	0.776
	2 +	4 (11%)	3 (11%)	
PD-L1-tumoral	Mean (min, max)	2.6% (0, 30%)	7.8% (0, 60%)	0.004
PD-L1-stromal	Mean (min, max)	1.9% (0, 15%)	7.3% (0, 50%)	0.007
CD8-tumoral	Mean (min, max)	7.4% (0, 50%)	11% (0, 40%)	0.004
CD8-stromal	Mean (min, max)	8.7% (0, 40%)	15% (0, 50%)	0.001
CD163-tumoral	Mean (min, max)	35.9% (0, 80%)	22.8% (5%, 50%)	0.003
CD163-stromal	Mean (min, max)	39.2% (10%, 80%)	24.8% (5%, 50%)	0.003

IHC immunohistochemistry

Table 3 Multivariable logistic regression model analysis of factors associated with response to neoadjuvant chemotherapy in 66 triple-negative breast carcinomas

Variable	Level	Incomplete response (n=38)	Complete response (n=28)	Multivariable analysis p value
Age	Mean (min, max)	53.2 (31, 74)	47 (26, 72.9)	0.226
Mitosis	1	3 (8%)	1 (4%)	0.031
	2	25 (66%)	12 (43%)	
	3	10 (26%)	15 (54%)	
PD-L1-tumoral	Mean (min, max)	2.6% (0, 30%)	7.8% (0, 60%)	0.340
PD-L1-stromal	Mean (min, max)	1.9% (0, 15%)	7.3% (0, 50%)	0.005
CD8-tumoral	Mean (min, max)	7.4% (0, 50%)	11% (0, 40%)	0.138
CD8-stromal	Mean (min, max)	8.7% (0, 40%)	15% (0, 50%)	0.125
CD163-tumoral	Mean (min, max)	35.9% (0, 80%)	22.8% (5%, 50%)	0.694
CD163-stromal	Mean (min, max)	39.2% (10%, 80%)	24.8% (5%, 50%)	<0.001

Discussion

Achieving pCR is associated with lower risk of progression and death, and better long-term survival outcomes in TNBC patients [25–27]. Tumor microenvironment components, such as TILs and PD-L1 expression on immune cells, play important roles in breast cancer patients' survival and the response to chemotherapy [4–6, 12, 13]. M2 macrophages, another important component within tumor microenvironment, promote tumor growth and are

associated with poor survival in breast cancer patients, especially in TNBC patients [14, 16, 18–23]. CD163 is a well-known specific marker for M2 macrophages. TNBCs usually show more CD163-positive TAMs than other subtypes of breast cancers [28]. Previous studies have demonstrated CD163 expression is correlated with poor prognosis in TNBC patients [19–21, 28, 29]. However, studies of M2 macrophages' effect on the response to NAC in breast cancer patients are lacking. To our knowledge, this is the first study to investigate the relationship between M2 macrophages and the response to NAC in TNBCs. Our data

have demonstrated that the presence of M2 macrophages, especially in stromal area, negatively affects the response to NAC in TNBCs.

In addition to M2 macrophages, several other factors including age, mitosis, CD8-positive T cells and PD-L1 expression were found to correlate with the response to NAC in our cohort. Univariate analysis identified younger age, increased CD8-positive TILs and increased PD-L1 expression as positive predictors for pCR, while multivariable analysis identified increased mitosis and increased PD-L1 expression in stromal immune cells as positive predictors for pCR. As mentioned above, previous studies have demonstrated a positive association between TILs and pCR. The failure to identify CD8-positive TILs as a significant predictor in multivariable analysis is probably caused by the small size of our study cohort, although the average percentage of CD8-positive cells in pCR group is greater than that in incomplete response group without statistically significant difference. The relationship between PD-L1 expression and cancer prognosis is controversial. PD-L1 expression is expected to be associated with a poorer prognosis in cancer patients due to its immune evasion mechanism to suppress the immune response to tumor cells. However, previous studies have demonstrated a positive association between PD-L1 expression and favorable prognosis. [10, 30, 31] However, this may be caused by the simple association of PD-L1 expression with increased TILs during anti-tumor immune response, rather than an association with tumor immune evasion in this setting. The positive association between PD-L1 expression and pCR found in current study may be simply explained as such.

Our study has several limitations. The greatest one is perhaps its small cohort size. Based on the findings from this relatively small cohort, we could not establish the positive relationship between CD8-positive T cells and pCR in multivariable analysis with statistical significance, although the univariate analysis showed a significant correlation. However, we were able to identify the presence of CD163-positive M2 macrophages as a negative predictor for pCR. The retrospective nature of the study and lack of long-term follow-up outcome are other limitations.

In summary, we have demonstrated a negative correlation between CD163-positive M2 macrophages and the response to NAC in TNBC patients, suggesting M2 macrophages' important role in predicting the response to NAC and/or potential target for developing future therapy.

Author contributions Study design: All. Data collection: VA, TS, HN, AVP, ZL. Data analysis: VA, TS, HN, ZL. Statistical oversight: LW. Manuscript preparation: VA, TS, HN, ZL. Manuscript revision: VA, TS, HN, AVP, ZL. Manuscript approval: All.

Funding None.

Declarations

Conflict of interest V. Arole, T. Shen, L. Wei, A. Parwani and Z. Li have no financial relationship to disclose. H. Nitta is an employee of Roche Tissue Diagnostics.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Research involving animal rights This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual patients included in the study.

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