Prognostic value of tumor-infiltrating lymphocyte subtypes in residual tumors of patients with triple-negative breast cancer after neoadjuvant chemotherapy

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Abstract
Background: After neoadjuvant chemotherapy (NAC), non-pathological complete response of breast cancer patients can benefit from tailored adjuvant chemotherapy. However, it is difficult to select patients with poorer prognosis for additional adjuvant chemotherapy to maximize the benefits. Our study aimed to explore whether the subtypes of tumor-infiltrating lymphocytes (TILs) in residual tumors (RT) is related to the prognosis of triple-negative breast cancer (TNBC) after NAC.

Methods: Data from patients with primary TNBC consecutively diagnosed at the Breast Disease Center of Peking University First Hospital from 2008 to 2014 were retrieved, and the cases with RT in the breast after NAC were enrolled. TILs subtypes in RT were observed by double-staining immunohistochemistry, and counted with the median TILs value per square millimeter as the cut-off to define high versus low TILs density in each subtype. The relationships between the TIL density of each subgroup and the clinicopathological characteristics of the RT after NAC patients were analyzed by Fisher exact test. Disease-free survival (DFS) and overall survival (OS) were analyzed by the Kaplan-Meier method and log-rank statistics.

Results: A total of 37 eligible patients were included in this study, and the median follow-up period was 50 months (range 17–106 months). There was no significant correlation between the infiltrate density of CD4+, CD8+, CD20+, and CD68+ lymphocytes and clinic-pathological characteristics. Significantly better prognosis was observed in patients with high CD4+-TILs (DFS: P = 0.005, OS: P = 0.021) and high CD8+-TILs (DFS: P = 0.018) and low CD20+-TILs (OS: P = 0.042). Further analysis showed that patients with CD4+/CD20+ ratio greater than 1 (DFS: P = 0.001, OS: P = 0.002) or CD8+/CD20+ ratio greater than 1 (DFS: P = 0.009, OS: P = 0.022) had a better prognosis.

Conclusions: Subtypes of TILs in RT is a potential predictive biomarker of survival in TNBC patients after NAC.

Keywords: Triple-negative breast cancer; Neoadjuvant chemotherapy; Residual tumors; Tumor-infiltrating lymphocyte subtypes

Introduction
Breast cancer is a heterogeneous tumor with different clinical and pathological features, biological behavior, response to treatment, and prognosis. Triple-negative breast cancer (TNBC) comprises 10% to 20% of all breast cancers, is usually composed of biologically aggressive and histologically high-grade tumors and is associated with a young age, advanced disease, and a high incidence of metastases and recurrence.1-3 Due to the lack of targeted treatment and the worst clinical outcome, TNBC is also an important challenge in today’s clinical practice. The National Comprehensive Cancer Network guidelines point out that neoadjuvant chemotherapy (NAC) is a preferred choice that can facilitate breast conservation, render inoperable tumors operable, and provide important prognostic information at an individual patient level based on the response to therapy, particularly in patients with TNBC.

Studies have confirmed a correlation between pathological complete response (pCR) and disease-free survival (DFS) or overall survival (OS) after NAC, and pCR has been proposed as a surrogate endpoint for the prediction of long-term clinical benefits, such as DFS and OS.4,5 Previous literature has pointed out that pCR can discriminate patients with good prognosis. In fact, the prognostic value of pCR is highest in human epidermal growth factor receptor 2-positive (non-luminal) and TNBC patients.6 Therefore, we have another problem:
how do we improve the long-term survival of non-pCR TNBC patients? The traditional view is that patients with TNBC are no longer treated after surgery. The CREATE-X study found that in the TNBC subgroup, the prognosis of patients who did not achieve pCR was significantly improved after post-operative intensive adjuvant therapy. However, the side effects in some patients are too strong to be tolerated. In the CREATE-X study, non-pCR patients were treated with an additional six or eight cycles of capecitabine, and 57.9% and 37.8%, respectively, completed the planned dose, whereas 23.9% and 36.7% had a dose reduction, and 18.2% and 25.4% discontinued capecitabine treatment. Thus, selecting a high-risk patient for additional intensive treatment was the focus of the current research.

Tumor-infiltrating lymphocytes (TILs) have also been shown to be predictive of the response to NAC. Denkert et al. first confirmed that TILs are associated with pCR in TNBC, and another study has produced consistent results. In addition, Loi et al. first proposed and confirmed that an increase in TILs was associated with a reduced risk of distant recurrence and death. A meta-analysis of the prognostic value of TILs in TNBC showed that tumors rich in TILs were associated with improved survival. Studies have also shown that a variety of TIL subtypes participate in the breast cancer immune response and subgroup cell cross-accommodation, jointly mediating and regulating tumor-associated immunity.

In recent years, a study showed that CD4+ and CD8+-TILs predict good prognosis in breast cancer patients. However, there have also been limitations to the studies of TILs in breast cancer, including the area ratio of TILs to the tumor stroma in hematoxylin and eosin staining; additionally, there is a lack of division of the TIL subtypes. The occurrence and development of tumors are highly correlated with the immune microenvironment. Although the development of most tumors is related to immune escape, the immune response induced by the tumor will complete the whole process of tumor development. Even in the late stage of cancer, the host’s anti-tumor immunity exists, and it can directly or indirectly affect the survival of patients.

It is necessary to investigate the tumor microenvironment and to explore the prognostic and predictive biomarker values of TIL subtypes present there. This is especially true for TNBC patients who have not achieved pCR after NAC. In this study, TNBC patients who did not achieve pathological complete remission after NAC were selected to explore the prognostic value of TIL subtypes in residual tumors. Immunohistochemistry was used to distinguish different subtypes of TILs. The immunohistochemistry-positive cell density was used to classify the infiltration of TILs. The feasibility of the study was previously confirmed.

Methods

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Ethical approval from the Institutional Ethics Examining Committee of Human Research of Peking University First Hospital (No. 2018-19) was obtained before patient recruitment. All participants signed a written informed consent form after a full explanation of the study was provided, including the schematic procedures and potential benefits and complications.

Patients

Between January 2008 and December 2014, a total of 3158 patients were newly diagnosed with breast cancer in the Breast Disease Center at Peking University First Hospital, and 324 patients received neoadjuvant therapy, of whom 74 (22.9%) were diagnosed with TNBC and were all treated with a six cycles of TA chemotherapy (cycled every 21 days: docetaxel 75 mg/m² or paclitaxel 135–175 mg/m², intravenous injection [iv], day 1; pirarubicin 50 mg/m² or epirubicin 75 mg/m², iv, day 1). The Collaborative Trials in Neoadjuvant Breast Cancer recommend defining pCR as a primary breast lesion and axillary lymph nodes without invasive cancer residue or residual carcinoma in situ (ypT0/Tis ypN0). According to the above definition, 24 TNBC patients achieved pCR: the pCR rate was 32.4%, and the other patients achieved non-pCR. Ultimately, a total of 37 of 50 patients completed clinical and follow-up data, and informed consent was obtained. The clinical, pathology, and follow-up data were complete for all these patients.

Immunohistochemistry and immunoactive cell count

Three-point five-micron sections were cut from the paraffin-embedded tissue block of the residual tumor, and immunohistochemistry for CD4+-TILs (clone EP204, dilution 1:100; Zhongshan Jinqiao Biotechnology Co., Ltd., China), CD8+-TILs (clone SP16, dilution 1:50; Zhongshan Jinqiao Biotechnology Co., Ltd.), CD20+-TILs (clone L26[2] dilution 1:150; DAKO, Denmark), and CD68+-TILs (clone PG-M1, dilution 1:200; Zhongshan Jinqiao Biotechnology Co., Ltd.) was performed. Using a double-blind method, the immunoreactive cell count was performed by two pathologists and averaged. In each slice, we selected five areas with the most abundant residual tumor interstitial lymphocytes in microscopic grids at 200× magnification and counted the number of immunoreactive cells within the specified area in microscopic grids at 400× magnification. The result was calculated based on the average cell numbers in these five areas. To define high versus low TIL patients, we used the median TIL value as the cut-off and divided the different subtypes into high and low groups.

Statistical analysis

All statistical analyses were performed by using the statistical software SPSS for Windows, version 24.0 (SPSS Inc., Chicago, IL, USA). In this study, the population of TIL density is a continuous natural number that satisfies the
normal distribution. We counted with the median TILs value per square millimeter as the cut-off to define high vs. low TILs density in each subtype. The relationship between the TIL density of each subgroup and clinicopathological characteristics (including age, menstrual status, tumor-node-metastasis [TNM] stage, histological grade, proliferation rate, node status, and the clinical efficacy of NAC) of the residual tumors after NAC was evaluated with Fisher exact test. The differences in DFS and OS between the high and low groups were analyzed with the Kaplan-Meier method and log-rank statistics. All analyses were two-sided, and a P-value <0.05 was considered statistically significant.

Results

Patient characteristics

Between 2008 and 2014, a total of 37 eligible patients were selected for our study. The patient characteristics are shown in Table 1. Clinical and pathological TNM classification (clinical TNM staging and pathological TNM staging) of patients were evaluated according to the Eighth Edition American Joint Committee on Cancer Staging criteria.[25] The pathological information of 37 patients was obtained by our pathologist according to the World Health Organization classification of tumors of the breast.[26]

Immunohistochemical staining of various TIL subpopulations in residual tumors

Immunohistochemical staining of various TIL subpopulations in residual tumors is shown in Figure 1. Among the 37 patients, the median number of TILs (count/mm²) for the four subtypes was as follows: CD4⁺-TILs, 1518 (range 281–6597), CD8⁺-TILs, 1585 (range 142–5357), CD20⁺-TILs, 970 (range 81–4355), and CD68⁺-TILs, 673 (range 188–2014).

Table 1: Relationships between the infiltration rate of TILs and clinicopathological features of the residual tumor after NAC, n = 37.

| Characteristics | CD4⁺ | CD8⁺ | CD20⁺ | CD68⁺ |
|-----------------|------|------|-------|-------|
|                 | High | Low | P     | High | Low | P     | High | Low | P     | High | Low | P     |
| Age (years)     |      |     |       |      |     |       |      |     |       |      |     |       |
| <35             | 3    | 1   | 0.584 | 1    | 2   | 1.00  | 1    | 2   | 1.00  | 1    | 2   | 1.00  |
| ≥35             | 34   | 17  | 0.254 | 19   | 15  | 0.352 | 13   | 21  | 0.103 | 17   | 17  | 0.013 |
| Menstrual status|      |     |       |      |     |       |      |     |       |      |     |       |
| Pre-menopausal  | 17   | 10  | 0.729 | 11   | 6   | 1.00  | 8    | 11  | 0.842 | 7    | 13  | 0.608 |
| Post-menopausal | 20   | 8   | 0.729 | 9    | 11  | 1.00  | 8    | 12  | 0.842 | 7    | 13  | 0.608 |
| cT              |      |     |       |      |     |       |      |     |       |      |     |       |
| cT1             | 3    | 2   | 1.00  | 1    | 2   | 1.00  | 1    | 2   | 1.00  | 0    | 3   | 0.304 |
| cT2             | 28   | 16  | 0.729 | 14   | 14  | 1.00  | 10   | 18  | 0.452 | 7    | 13  | 0.608 |
| cT3             | 2    | 1   | 1.00  | 0    | 2   | 1.00  | 1    | 1   | 1.00  | 1    | 1   | 1.00  |
| cT4             | 4    | 1   | 1.00  | 1    | 3   | 1.00  | 3    | 1   | 1.00  | 2    | 2   | 1.00  |
| cN              |      |     |       |      |     |       |      |     |       |      |     |       |
| cN0             | 21   | 12  | 0.254 | 4    | 17  | 0.940 | 10   | 11  | 0.254 | 10   | 11  | 0.254 |
| cN1             | 10   | 5   | 0.940 | 3    | 7   | 0.940 | 6    | 4   | 0.841 | 3    | 7   | 0.841 |
| cN2             | 4    | 2   | 0.236 | 1    | 3   | 1.00  | 2    | 2   | 1.00  | 0    | 4   | 0.236 |
| cN3             | 2    | 1   | 1.00  | 2    | 0   | 1.00  | 1    | 1   | 1.00  | 0    | 2   | 1.00  |
| Grade of primary tumors |      |     |       |      |     |       |      |     |       |      |     |       |
| 1               | 1    | 1   | 1.00  | 1    | 0   | 1.00  | 0    | 1   | 1.00  | 0    | 1   | 1.00  |
| 2               | 9    | 4   | 0.236 | 3    | 6   | 0.236 | 5    | 4   | 0.236 | 2    | 7   | 0.236 |
| 3               | 27   | 15  | 1.00  | 6    | 21  | 1.00  | 13   | 14  | 1.00  | 11   | 16  | 1.00  |
| Ki-67 (%) of primary tumors |      |     |       |      |     |       |      |     |       |      |     |       |
| ≤14             | 1    | 1   | 1.00  | 1    | 0   | 1.00  | 1    | 0   | 1.00  | 1    | 1   | 1.00  |
| >14             | 36   | 19  | 0.452 | 9    | 27  | 0.452 | 18   | 18  | 0.452 | 13   | 23  | 0.452 |
| Clinical efficacy of NAC |      |     |       |      |     |       |      |     |       |      |     |       |
| CR              | 2    | 0   | 0.420 | 2    | 0   | 0.420 | 1    | 1   | 0.420 | 1    | 1   | 0.420 |
| PR              | 26   | 14  | 0.420 | 15   | 11  | 0.420 | 10   | 16  | 0.420 | 14   | 12  | 0.420 |
| SD              | 8    | 3   | 0.536 | 4    | 4   | 0.536 | 3    | 5   | 0.536 | 2    | 6   | 0.536 |
| PD              | 1    | 1   | 0.310 | 1    | 0   | 0.310 | 1    | 1   | 0.310 | 1    | 1   | 0.310 |
| Evaluation after NAC |      |     |       |      |     |       |      |     |       |      |     |       |
| G1              | 8    | 3   | 0.077 | 3    | 5   | 0.077 | 2    | 6   | 0.077 | 4    | 4   | 0.077 |
| G2              | 6    | 1   | 0.536 | 1    | 5   | 0.536 | 1    | 5   | 0.536 | 3    | 3   | 0.536 |
| G3              | 17   | 9   | 0.536 | 11   | 6   | 0.536 | 8    | 9   | 0.536 | 9    | 8   | 0.536 |
| G4              | 6    | 5   | 0.268 | 5    | 1   | 0.268 | 3    | 3   | 0.268 | 2    | 4   | 0.268 |
| pT              | 0.658 | 0.310 | 0.268 | 0.658 | 0.310 | 0.268 | 0.749 | 0.749 | 0.749 |
| pT1             | 12   | 6   | 0.749 | 6    | 6   | 0.749 | 3    | 9   | 0.749 | 7    | 5   | 0.749 |

(continued)
and CD68⁺-TILs, 969 (range 125–1948). Then, according to the median value, the different subtypes were divided into high and low groups for subsequent analyses.

**Association of the TIL density in each subgroup with the clinicopathological parameters**

The association of the TIL density in each subgroup with the clinicopathological parameters (including age, menstrual status, TNM stage, histological grade, proliferation rate, node status, and the clinical efficacy of NAC) of the residual tumors after NAC was evaluated with Fisher exact test. The clinical and pathological efficacies of NAC were judged according to the response evaluation criteria in solid tumors (v1.1) and Miller-Payne criteria. The results are summarized in Table 1. The results showed no significant correlation between the TIL subtypes and the clinicopathological features of the residual tumor, indicating that the TIL subtype can be used as an independent predictor.

**Infiltration ratio and prognosis of TIL subtypes in the residual tumors of TNBC patients after NAC**

**DFS and OS of all enrolled patients**

The follow-up data of 37 patients were complete. The final follow-up visit occurred in June 2016. The median follow-up time was 50 months (range 17–106 months). Seven patients had recurrence and metastasis, and five died. DFS was defined as the total time from surgery to the first recurrence or metastasis of the disease. OS was defined as the total time from the first diagnosis of breast cancer to death. The 5-year DFS rate was 81.0%, and the 5-year OS rate was 86.3%. The total outcomes are shown by the Kaplan-Meier survival analysis in Figure 2.

**Prognostic value of TILs in residual tumors of enrolled patients**

Analysis of the correlation between the infiltration ratio of TILs and DFS or OS by the Kaplan-Meier survival analysis.
The univariate factor analysis showed that CD4+ lymphocyte infiltration was significantly positively correlated with DFS and OS, and high CD4+-TILs signified improved survival (DFS: $P = 0.005$, OS: $P = 0.021$). CD8+ lymphocyte infiltration was positively correlated with DFS ($P = 0.018$). CD20+ lymphocyte infiltration was negatively correlated with OS, and low CD20+-TILs signified longer DFS ($P = 0.042$). However, the CD68+-TILs showed no significant correlation with a long-term benefit [Figure 3].

Then, we calculated the ratio of CD4+ and CD20+ and the ratio of CD8+ and CD20+ based on this definition of the TIL ratios and subsequently divided them into two groups according to whether the value was greater than 1. Then, the survival function was used to compare the differences. The results showed that significantly improved long-term survival was observed in the high CD4+/CD20+ (DFS: $P = 0.001$, OS: $P = 0.002$) and high CD8+/CD20+ (DFS: $P = 0.009$, OS: $P = 0.022$) groups. There was no correlation between the infiltration ratio of CD4+, CD8+, CD20+,
and CD68+ TILs and the clinicopathological features of the residual tumor after NAC [Figure 4].

Discussion

The tumor microenvironment is a dynamic ecosystem composed of tumor cells and various non-cancer cells present in the extracellular matrix. More and more studies show that tumor microenvironment is one of the driving factors of tumor progression and invasion. TILs constitute the tumor immune microenvironment, which influences the effects of immunotherapy and the effects of anti-cancer drugs, as well as treatment outcomes.

The univariate analysis of the relationship between the TIL infiltration ratio and DFS or OS found that high CD4+ lymphocyte infiltration; high CD8+ lymphocyte infiltration and low CD20+ lymphocyte infiltration predicts a better outcome. There was no significant correlation between the CD68+ TIL subtypes and long-term benefits; however, the Kaplan-Meier curve showed a significant difference trend. This finding may be due to the small number of patients enrolled and the small number of events during follow-up.

Tumor antigens can trigger adaptive immune responses and perform immune surveillance functions to destroy tumor cells. Activated T lymphocytes, which are composed of CD4+ and CD8+ T cells, are the most important effector T cells. CD4+ T cells are mostly helper T lymphocytes, which assist with humoral immunity and cellular immunity. Such cells cannot directly recognize and kill tumor cells but rely on antigen-presenting cells to participate in the activation of other immune cells by recognizing the soluble antigen secreted by tumor cells, thereby exerting an anti-tumor effect. CD4+ T cells in breast cancer can increase the number and function of infiltration of other tumor killer cells and have been shown to be associated with improved survival benefits in the field of adjuvant therapy. In this study, CD4+ T cells were significantly positively correlated with DFS and OS, confirming that CD4+ T cells play a role in predicting good prognosis in neoadjuvant therapy.

CD8+ T cells, or tumor killer T cells, are the most important effector cells in tumor-associated immune responses. Studies have shown that CD8+ T cell density in breast cancer is associated with a good prognosis. The univariate analysis in this study showed a correlation between CD8+ T cells and good long-term prognosis, consistent with literature reports.

B lymphocytes are another major component of lymphocytes that secrete immunoglobulins and complete humoral immunity. The CD20 molecule expressed by its cell membrane can serve as a molecular marker specific for B lymphocytes. At present, the role of B lymphocytes in the anti-tumor mechanism has not been elucidated, especially for the study of B lymphocytes and prognosis before and after NAC for breast cancer. There was a significant negative correlation between CD20+ cells and DFS in this study, but there was no statistically significant correlation with OS. However, the survival curve showed a negative correlation trend. Further research and verification are needed.

Tumor-associated macrophages (TAMs) are another important component of breast tumor TILs. Their main functions include clearing cell debris and initiating an immune response. According to the phenotype and secreted cytokines, TAMs can be divided into two types: classical activated M1 cells and selectively activated M2 cells. The two polarization types play opposite roles in tumor immune responses. The most specific marker is CD68+, as TAMs reflect the degree of TAM infiltration in tumor tissues. Clinical studies have shown that the amount of TAMs infiltrated in the tumor microenvironment is associated with a poor prognosis in breast cancer, but studies on the efficacy and prognosis of TAMs before and after NAC for breast cancer patients are rare. This study also failed to demonstrate the predictive value of TAMs for the prognosis of breast cancer patients after NAC.

Our study has several limitations, we only selected 37 cases of TNBC for exploration. Although the number of cases is small, we compared all clinical and pathological information before and after neoadjuvant therapy. There were
Figure 3: Kaplan-Meier curves of outcomes for different TILs subtypes. Kaplan-Meier analysis of disease-free survival and CD4+ TILs (A), CD8+ TILs (B), CD20+ TILs (C), and CD68+ TILs (D); and Kaplan-Meier analysis of overall survival (OS) and CD4+ TILs (E), CD8+ TILs (F), CD20+ TILs (G), and CD68+ TILs (H). TIL: Tumor-infiltrating lymphocyte.
significant statistical differences between the high TILs density and the low TILs density subgroup. Therefore, it is necessary to continue to expand the sample size for confirmation in subsequent studies.

In conclusion, the infiltration ratio of CD4+ and CD8+ TILs was significantly positive, and CD20+ TILs negatively correlated with TNBC patient survival after NAC. In addition, patients with a CD4+/CD20+ ratio or a CD8+/CD20+ ratio greater than 1 had a good prognosis, which indicated that the combined analysis of TIL subgroups may better refine the prognostic stratification.

**Conflicts of interest**

None.

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