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Impact of tumor-infiltrating lymphocytes on pathological complete response after neoadjuvant chemotherapy in patients with early triple-negative breast cancer

Utjecaj tumor-infiltrirajućih limfocita na kompletni patološki odgovor nakon neoadjuvantne kemoterapije u bolesnica s ranim trostruko negativnim rakom dojke

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Summary

Description of the subject: Triple-negative breast cancer (TNBC), a breast cancer subtype, is characterized by the lack of both estrogen and progesterone hormonal receptors expression and by the absence of human epidermal growth factor receptor 2 overexpression. Patients with a pathological complete response (pCR) have better disease-free and overall survival compared to those with residual disease. The high level of tumor-infiltrating lymphocytes (TILs) is associated with a higher response to neoadjuvant chemotherapy (NAC) and better prognosis.

Objective: Evaluation of TILs and their predictive impact in early TNBC in an Algerian population.

Methods: We assessed TILs and correlated them with the pCR rate in 94 early TNBC patients treated from 2015 to 2017 who underwent breast microbiopsy, NAC, and then surgery.

Results: Among 94 early TNBC patients, 53 (56.4%) achieved pCR and 41 (43.6%) had a residual disease. While some clinicopathological factors did not affect pCR, stromal TILs showed significant correlation with pCR ($P < 0.0001$). The presence of $CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$ TILs was also significantly correlated with pCR ($P < 0.0001$, $P = 0.001$, $P = 0.0003$ and $P = 0.0001$, respectively).

Conclusion: Our data showed that TILs were significantly associated with pCR, suggesting that TILs are a predictive biomarker for pCR in early TNBC patients treated by NAC in our cohort.

Key words: triple-negative breast cancer, pathological complete response, tumor-infiltrating lymphocytes, neoadjuvant chemotherapy, predictive biomarker.

Sažetak

Opis teme: Trostruko negativni rak dojke (TNBC), podtip raka dojke, okarakteriziran je izostankom ekspresije hormonalnih receptora i estrogena i progesterona te izostankom prekomjerne ekspresije receptora epidermalnog čimbenika rasta 2. Bolesnice s kompletnim patološkim odgovorom (pCR) imaju bolje preživljavanje bez bolesti i općenito preživljavanje u usporedbi s onima s rezidualnom bolešću. Visoka razina tumor-infiltrirajućih limfocita (TIL-i) povezana je s boljim odgovorom na neoadjuvantnu kemoterapiju (NAC) i s boljom prognozom.

Cilj: Procijeniti TIL-e i njihov predvidivi učinak na rani TNBC u populaciji u Alžiru.

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Metode: Procijenili smo TIL-e i korelirali ih s postotkom pCR-a u 94 bolesnica s ranim TNBC liječenih od 2015. do 2017. godine, koje su bile podvrgnute mikrobiopsiji dojki, neoadjuvantnoj kemoterapiji i potom operativnom zahvatu.

Rezultati: Među 94 bolesnice s ranim TNBC, 53 njih (56,4 %) postiglo je pCR, a 41 (43,6 %) je imala rezidualnu bolest, dok neki kliničko-patološki čimbenici nisu utjecali na pCR, stromalni TIL-i pokazali su značajnu korelaciju s pCR-om (P < 0,0001). Prisutnost CD3+, CD4+, CD8+ i CD20+ TIL-a značajno je korelirala i s pCR-om (P < 0,0001, P = 0,001, P = 0,0003, odnosno P = 0,0001).

Zaključak: Naši podaci pokazali su da su TIL-i značajno povezani s pCR-om, što navodi na zaključak da su TIL-i prediktivni biomarker za pCR u bolesnica s ranim TNBC liječenima neoadjuvantnom kemoterapijom u našoj kohorti.

Ključne riječi: trostruko negativni rak dojke, patološki potpuni odgovor, limfociti koji infiltriraju tumor, neoadjuvantna kemoterapija, prediktivni biomarker.

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Introduction

Triple-negative breast cancer (TNBC) is a breast cancer subgroup characterized by the lack of expression of human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and estrogen receptor (ER) assessed by immunohistochemistry (IHC). It represents 15% to 20% of breast carcinomas.[1] TNBC is a type of breast cancer that affects younger women more often, and generally has a worse prognosis than other types of breast cancer.[2] This subtype is usually aggressive, poorly differentiated, and is characterized by a high mitotic index.[1] TNBC is heterogeneous in terms of molecular profile. Lehmann et *al*.[3] evaluated the gene expression profiles of 587 TNBC. Six molecular subtypes have been identified including 2 basal-like, a mesenchymal, a mesenchymal stem-like, an immunomodulatory, and a luminal androgen receptor subtype. For each molecular subtype, treatment sensitivity varies from a subtype to another.

Nowadays, neoadjuvant chemotherapy (NAC) is considered as the standard care in early TNBC.[4] In early breast cancer, patients who achieved a pathological complete response (pCR) after NAC tend to have improved survival compared to patients with residual disease.[5]

Both innate and adaptive immunity play a principal role in the immune surveillance against cancer and can limit cancer progression.[6] Previous research has shown that stromal TILs were an independent predictive and prognostic biomarker of outcome in early TNBC.[7] Studies have explored the predictive and prognostic value of TILs subsets in early breast cancer. For instance, previous studies have shown that the increased infiltration of $CD3^+$ and $CD20^+$ TILs has significantly predicted pCR.[8,9] Other studies have also revealed that $CD4^+$ and $CD8^+$ TILs were found to be predictive of pCR[9,10] and had a favorable prognostic value.[11]

In our research, we assessed TILs and their impact on pCR rate in early TNBC in Algerian patients.

Material and methods

Studied population

The study cohort included 94 patients treated for stage I-III breast cancer from 2015 to 2017 in Pierre and Marie Curie Cancer Center (CPMC) in Algiers-Algeria. All patients had a core microbiopsy confirming a TNBC subtype. They received NAC based on 3 cycles; combining docetaxel and carboplatin every three weeks followed by three-cycle-anthracycline-based chemotherapy. The study was performed according to the ethical recommendations of the Helsinki declaration. An informed and written consent dated and signed by the patients was accordingly obtained by our ethical committee.

Clinicopathological factors assessment

The assessed clinicopathologic factors included: patients' age at diagnostic, tumor stage, nodal status, tumor-node-metastasis (TNM) stage, histological type, histological grade, biomarkers (ER, PR, HER2 and Ki-67 proliferation index), and histopathological response. Tumor stage, nodal status, and TNM stage were clinically evaluated using pre-treatment images in compliance with the Union for International Cancer Control TNM classification.[12] As for the histological type, the histological grade, as well as the biomarkers, were determined using pre-therapeutic microbiopsies specimens. Histological types were defined as per the WHO classification.[13] While the histological grade was assessed using the Scarff, Bloom and Richardson method,[14] IHC of ER and PR was performed using ER antibodies (clone SP1, ready-to-use, Ventana) and PR antibodies (clone 1E2, ready-to-use, Ventana). The ER and PR expression was assessed by the Allred Immunohistochemistry Score.[15] Concerning the HER2 expression, it was performed using a HER2 antibody (clone 4B5, ready-to-use, Ventana). HER2 2+ cases were analyzed by dual in situ hybridization using an

INFORM HER2 Dual ISH DNA Probe Cocktail (Ventana). HER2 expression and in situ hybridization were both assessed following the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines for HER2 testing.[16] The Ki-67 proliferation index was evaluated by IHC using a Ki-67 antibody (clone 30-9, ready-to-use, Ventana). The result was given in percentage. All the four-marker-analysis was carried out on the Ventana BenchMark ULTRA automated platform (BenchMark ULTRA automates, UltraView Universal DAB Detection Kit, Ventana Medical Systems; Tucson, AZ, USA).

In our study, the pCR was defined as a non-residual invasive tumor in both the breast and the axillary lymph nodes, based on the histopathological evaluation of the resection specimen after NAC. Residual carcinoma in situ was included in the pCR (ypT0/Tis ypN0).

Tumor-infiltrating lymphocytes assessment

Pre-therapeutic core microbiopsies were formalin-fixed and paraffin-embedded. Thin sections were cut and stained with hematoxylin and eosin (H&E). Stromal TILs evaluation was performed on H&E sections on core microbiopsies. Stromal TILs were defined as mononuclear cells localized in the stroma surrounding carcinoma cells and were assessed according to international TILs working group guidelines (stromal TILs were defined as the percentage of tumor stroma area infiltrated by lymphocytes).[17] Regarding the evaluation of stromal TILs subpopulations, formalin-fixed and paraffin-embedded sections of core microbiopsies were stained using an IHC method. The following antibodies were used on IHC analysis: CD3 (IR503 polyclonal antibody, ready-to-use, Dako), CD4 (clone 4B12, ready-to-use, Dako), CD8 (clone C8/144B, ready-to-use, Dako) and CD20 (clone L26, ready-to-use, Dako). The analysis of all these markers was carried out under the Ventana BenchMark ULTRA automated platform (BenchMark ULTRA automates, UltraView Universal DAB Detection Kit, Ventana Medical Systems; Tucson, AZ, USA). TILs subsets were reported as the percentages of tumor stroma area infiltrated by cells positive for CD3, CD4, CD8, and CD20.

In this study, a semi-quantitative scoring method was utilized to evaluate the density of stromal TILs and TILs subsets. We categorized the findings into three groups: low (score: 0-10%), intermediate (score: 11-50%) and high (score: 51-100%).

Statistical analysis

All clinicopathological factors (age at diagnosis, tumor stage, nodal status, TNM stage, histological type, Ki-67 proliferation index, histological grade, stromal TILs and TILs subsets) were compared between the pCR and non-pCR groups. The relationship between clinicopathological factors and pCR was determined using the chi-squared test for categorical variables and the Welch t-test for continuous variables. A P value < 0.05 was considered statistically significant. The calculations were performed using the R statistical software, version 3.5.

Results

Clinicopathological characteristics

Patients' clinicopathological characteristics are shown in Table 1. The mean and median age was 40.8 ± 9.528 years and 38.5 years, respectively (range, 22-64 years). Most patients were diagnosed with T2 (31.9%) and T3 (41.5%) tumors, whereas T1 and T4 diseases were seen in only 1.1% and 25.5% respectively.

The axillary lymph nodes metastasis was present in 60.6%. In terms of the TNM stage, only one case (1.1%) was classified as stage I, 47 cases (50%) as stage II, and 46 cases (48.9%) as stage III. Invasive ductal carcinoma was the predominant histological type (97.9%).

The mean rate of the Ki-67 proliferation index was 61.82 ± 22.95% (range, 10-90%). The histological grade was grade II in 36 cases (38.3%) and grade III in 58 cases (61.7%). Among 94 patients, 56.4% (54 patients) achieved pCR, whereas 43.6% (41 patients) still had residual disease.

As far as stromal TILs are concerned, 19 cases (20.2%) had low TILs, 50 cases (53.2%) had intermediate TILs and 25 cases (26.6%) had high TILs.

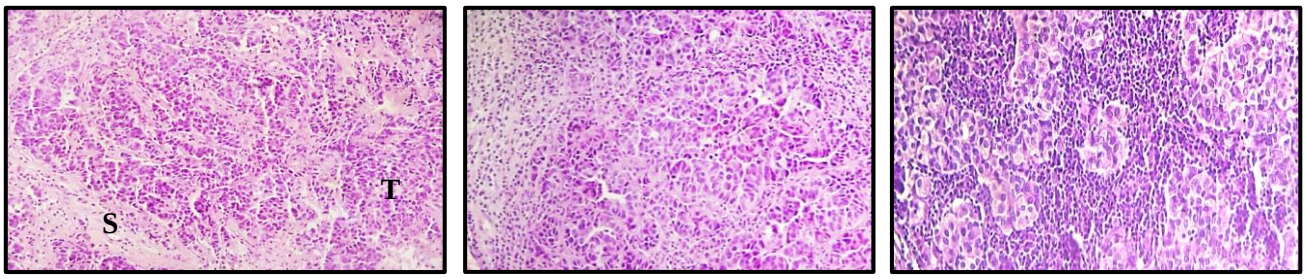
For stromal $CD3^+$ TILs, 19 cases (20.2%) had low $CD3^+$ TILs, 54 cases (57.5%) had intermediate $CD3^+$ TILs and 21 cases (22.3%) had high $CD3^+$ TILs. When it comes to stromal $CD4^+$ TILs, 43 cases (45.7%) had low $CD4^+$ TILs, 48 cases (51.1%) had intermediate $CD4^+$ TILs and 3 cases (3.2%) had $CD4^+$ high TILs. For stromal $CD8^+$ TILs, 31 cases (33%) had low $CD8^+$ TILs, 53 cases (56.4%) had intermediate $CD8^+$ TILs and 10 cases (10.6%) had high $CD8^+$ TILs. Finally, stromal $CD20^+$ TILs, 72 cases (76.6%) had low $CD20^+$ TILs and 22 cases (23.4%) had intermediate $CD20^+$ TILs. No high $CD20^+$ TILs infiltrate was seen.

Representative microphotographs showing different stromal TILs densities by H&E and IHC staining in the examined pre-therapeutic microbiopsies specimens are shown in Pictures 1 and 2.

Table 1 Clinicopathological factors of all patients
Tablica 1. Kliničko patološki čimbenici svih bolesnika

Clinicopathological factors *Kliničkopatološki čimbenici*	Number of patients No. (%) *Broj bolesnika* (n = 94)
Age at diagnosis (years) / *Dob na dijagnozi (godine)* Mean ± SD/ *Srednje ± SD* Range/ *Domet*	 40.8 ± 9.528 22-64
Tumor stage / *Stadij tumora* T1 T2 T3 T4	 1 (1.1) 30 (31.9) 39 (41.5) 24 (25.5)
Nodal status / *Nodalni status* Negative / *Negativan* Positive / *Pozitivan*	 37 (39.4) 57 (60.6)
TNM stage / *TNM faza* I II III	 1 (1.1) 47 (50) 46 (48.9)
Histological type / *Histološki tip* Ductal / *Duktalni* Other / *Ostalo*	 92 (97.9) 2 (2.1)
Ki-67 proliferation index / *Indeks proliferacije* Ki-67 (%) Mean ± SD / *Srednje ± SD* Range / *Domet*	 61.82 ± 22.95 10-90
Histological grade / *Histološka ocjena* II III	 36 (38.3) 58 (61.7)
Histopathological response / *Histopatološki odgovor* pCR Non-pCR/ *Nije pCR*	 53 (56.4) 41 (43.6)
Stromal TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 19 (20.2) 50 (53.2) 25 (26.6)
Stromal $CD3^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / Visoko	 19 (20.2) 54 (57.5) 21 (22.3)
Stromal $CD4^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / Visoko	 43 (45.7) 48 (51.1) 3 (3.2)
Stromal $CD8^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 31 (33) 53 (56.4) 10 (10.6)
Stromal $CD20^+$ TILs Low / *Nisko* Intermediate / *Srednje*	 72 (76.6) 22 (23.4)

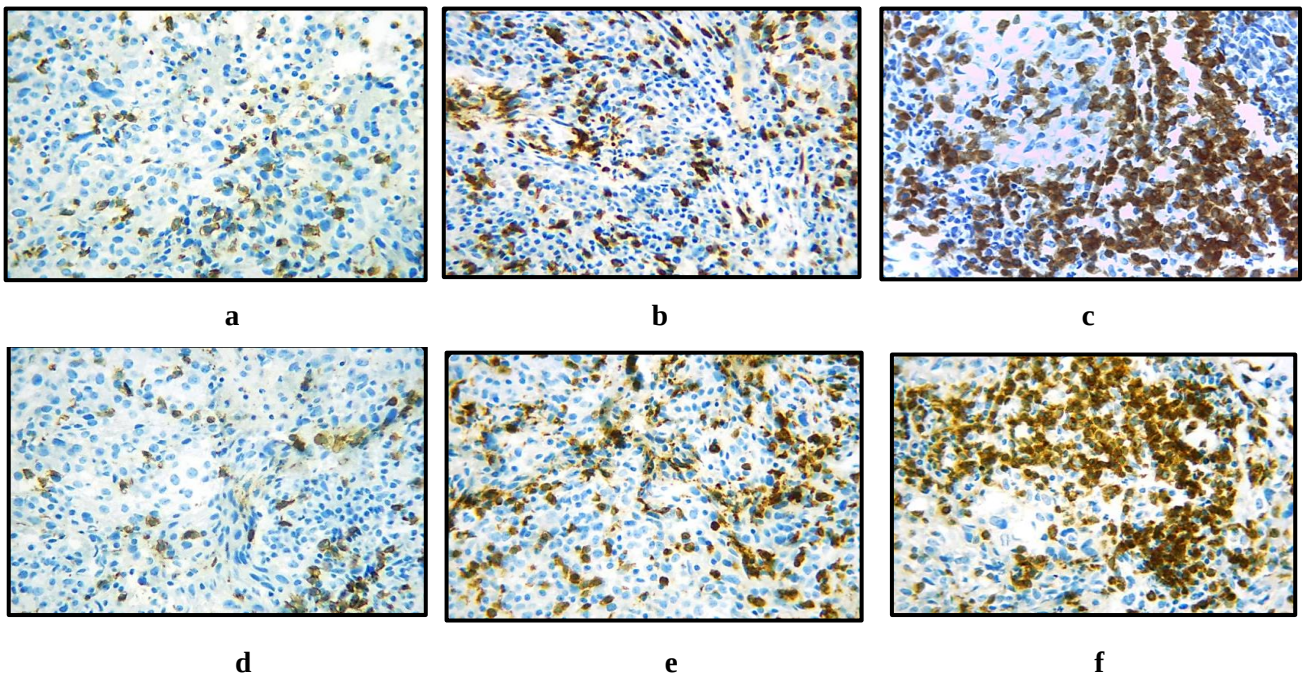
SD: standard deviation, TNM: tumor-node-metastasis, pCR: pathological complete response, TILs: tumor-infiltrating lymphocytes./ *SD: standardna devijacija, TNM: metastaza u tumoru-čvoru, pCR: potpuni patološki odgovor, TIL: limfociti koji infiltriraju tumor.*



a **b** **c**

Picture 1 Representative microphotographs of stromal TILs according to TILs density (H&E staining, x200). (a) Low TILs, (b) Intermediate TILs, (c) High TILs. T: tumor; S: stroma.

Slika 1. Reprezentativne mikrofotografije stromalnih TILs prema gustoći TILs (bojanje H&E, x200). (a) Niski TIL, (b) Srednji TIL, (c) TIL. T: tumor; S: stroma.



a **b** **c**

d **e** **f**

Picture 2 Immunohistochemical staining of stromal TILs (x400). (a) Low $CD3^+$ TILs, (b) Intermediate $CD3^+$ TILs, (c) High $CD3^+$ TILs, (d) Low $CD8^+$ TILs, (e) Intermediate $CD8^+$ TILs, (f) High $CD8^+$ TILs.

Slika 2. Imunohistokemijsko bojanje stromalnih TIL-ova (x400). (a) Niski CD3 + TIL, (b) Srednji CD3 + TIL, (c) CD3 + TIL, (d) Niski CD8 + TIL, (e) Srednji CD8 + TIL, (f) Visoki CD8 + TIL.

The relationship between clinicopathological factors and pCR

All parameters, including patients' age at diagnostics, tumor stage, nodal status, TNM stage, histological type, Ki-67 proliferation index, histological grade, stromal TILs and TILs subsets, were compared between the pCR and non-pCR groups (Table 2).

There was no significant relationship between pCR and age at diagnosis (P = 0.341), tumor stage (P = 0.371), nodal status (P = 0.562), TNM stage (P = 0.856), histological type (P = 1), Ki-67 proliferation index (P = 0.806) and histological grade (P = 0.200).

Increased infiltration of stromal TILs was significantly associated with pCR (P < 0.0001). A significant association was found between pCR and higher pre-NAC infiltration by stromal $CD3^+$ T cells (P < 0.0001), stromal $CD4^+$ T cells (P = 0.001), stromal $CD8^+$ T cells (P = 0.0003) and stromal $CD20^+$ B cells (P = 0.0001).

Table 2 Comparison of responding and non-responding patients' clinicopathological factors
Tablica 2. Usporedba kliničko patoloških čimbenika pacijenata koji reagiraju i koji ne reagiraju

Clinicopathological factors *Kliničko patološki čimbenici*	pCR (n = 53) No. (%)	Non-pCR No. (%) *Nije pCR* (n = 41)	*P* Value P *Vrijednost*
Age at diagnostic (years) / *Dob na dijagnostici (godine)* Mean ± SD / *Srednje ± SD*	39.96 ± 9.19	41.88 ± 9.958	0.341
Tumor stage / *Stadij tumora* T1-2 T3-4	 20 (37.74%) 33 (62.26%)	 11 (26.83%) 30 (73.17%)	0.371
Nodal status / *Nodalni status* Negative / *Negativan* Positive / *Pozitivan*	 19 (35.85%) 34 (64.15%)	 18 (43.9%) 23 (56.1%)	0.562
TNM stage / *TNM faza* I-II III	 28 (52.83) 25 (47.17)	 20 (48.78) 21 (51.22)	0.856
Histological type / *Histološki tip* Ductal / *Duktalni* Other / *Ostalo*	 52 (98.11) 1 (1.89)	 40 (97.56) 1 (2.44)	1
Ki-67 proliferation index / *Indeks proliferacije Ki-67 (%)* Mean ± SD / *Srednje ± SD* Range / *Domet*	62.34 ± 21.99	61.15 ± 24.41	0.806
Histological grade / *Histološka ocjena* II III	 17 (32.08%) 36 (67.92%)	 19 (46.34%) 22 (53.66%)	0.200
Stromal TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 4 (7.5%) 26 (49.1%) 23 (43.4%)	 15 (36.6%) 24 (58.5%) 2 (4.9%)	< 0.0001
Stromal $CD3^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 4 (7.5%) 29 (54.7%) 20 (37.8%)	 15 (36.6%) 25 (61%) 1 (2.4)	< 0.0001
Stromal $CD4^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 16 (30.2%) 34 (64.1%) 3 (5.7%)	 27 (65.9%) 14 (34.1%) 0 (0%)	0.001
Stromal $CD8^+$ TILs Low / *Nisko* Intermediate / *Srednje* High / *Visoko*	 10 (18.9%) 33 (62.2%) 10 (18.9%)	 21 (51.2%) 20 (48.8%) 0 (0%)	0.0003
Stromal $CD20^+$ TILs Low / *Nisko* Intermediate / *Srednje*	 34 (64.15%) 19 (35.85%)	 38 (92.68%) 3 (7.32%)	0.0001

pCR: pathological complete response, SD: standard deviation, TNM, tumor-node-metastasis, TILs: tumor- infiltrating lymphocytes / *pCR: potpuni patološki odgovor, SD: standardna devijacija, TNM, metastaza tumorskih čvorova, TIL: limfociti koji infiltriraju tumor*

Discussion

To our knowledge, this is the first publication evaluating lymphocytic infiltrate and its impact on pCR in Algerian patients treated for early TNBC. This study differs from previous investigations in that it classified TILs into three groups, to get a clearer comprehension of their relationship with the response to NAC.

The development of cancer cells is characterized by interactions of tumor cells with the tumor micro-

environment, such as tumor-infiltrating immune cell populations.[18] In the TNBC case, infiltrated immune cells are found in up to 75% of tumor, with up to 20% of the tumor having an especially high infiltration.[19]

In our study, we found out that increased levels of stromal TILs showed a significantly higher pCR rate. Similar to our findings, the GeparSixto study has shown that stromal TILs were independently and significantly associated with a high pCR rate in early TNBC.[20] Besides, the role of stromal TILs in different subsets of breast cancer was stressed by Denkert et *al*.[7] reporting that as an immunological marker, stromal TILs were an independent predictor of pCR in all breast cancer subtypes (triple-negative, HER2-positive, and luminal-HER2-negative early breast cancer) and were also associated with a favorable prognosis in triple-negative and HER2-postive early breast cancer.

The impact of TILs subsets on response to NAC is less explored in early TNBC and there are only limited studies that have investigated the relationship between some TILs subsets and pCR in purely early TNBC cohorts.

What was noticed was that the high level of stromal $CD3^+$ TILs was significantly associated with a high pCR rate. In the tumor microenvironment, TILs tend to be mainly $CD3^+$ T lymphocytes,[21] and the majority are $CD3^+/CD8^+$ T lymphocytes.[22,23] Previous investigations have shown that stromal $CD3^+$ TILs were found to be a predictive factor for pCR.[8-10]

Our study also revealed that stromal $CD4^+$ TILs were significantly associated with a high pCR rate. Previous studies have shown that $CD4^+$ TILs indicated a good response to NAC and a favorable prognosis in early breast cancer patients. For instance, one study has shown that the presence of stromal $CD4^+$ TILs had an independent predictive value for pCR.[10] Further studies have suggested that a high level of $CD4^+$ T cells in the breast tumors was a good prognostic indicator.[11,24] CD4 is expressed in many T cell subsets including T helper 1 cells, T helper 2 cells, T helper 17 cells, regulatory T cells, and T follicular helper cells, each of which may have a different role on pCR and prognosis. T helper 1 (Th1) cells involved in the antitumor immune response support $CD8^+$ T lymphocytes by secreting interferon-γ and interleukin-2.[25] Gu-Trantien et *al*.[21] reported that Th1 subset was associated with a better response to NAC in all breast cancer subgroups assessed (ER-negative/HER2-negative, ER-positive/HER2-negative, and HER2-positive early breast cancer) and with a good prognosis in patients with early HER2-positive breast cancer. The presence of Treg cells was linked with both bad and good.[26-28] Oda et *al*.[29] demonstrated that the presence of large numbers of intratumoral Treg cells into pre-therapeutic biopsy specimens of early breast cancer patients was an independent predictive factor for pCR. Th17 cells appear to have both antitumor and protumor effects.[25] In a study on early breast cancer, the presence of Th17 cells was correlated with a prognosis improvement.[30]

In our study, we demonstrated that stromal $CD8^+$ TILs were significantly associated with the higher pCR rate. Upon exposure to foreign and tumor antigens, $CD8^+$ T cells differentiate into $CD8^+$ cytotoxic T lymphocytes (CTLs) and play a key role in the tumor-specific cellular adaptive immune response. $CD8^+$ CTLs induce apoptosis in tumor cells through death ligands and perforin/granzyme dependent pathways.[31] Studies are examining the role of $CD8^+$ TILs in breast cancer. Denkert et *al*.[20] showed that high mRNA expression of CD8A was a significant predictor of pCR in triple-negative and HER2-positive early breast cancer. Also, it was reported that a high level of $CD8^+$ TILs in early breast cancer was an independent predictive factor of pCR,[32] and was associated with a good prognosis.[11,33]

We discovered that tumor infiltration by stromal $CD20^+$ TILs was significantly higher in breast cancer patients who achieved a pCR than in those who did not achieve a pCR. B cells have antitumor effects through several pathways, including the presentation of tumor antigens and stimulation of anti-tumor responses of $CD8^+$ and $CD4^+$ T cells, secretion of anti-tumor auto-antibodies that can promote cancer cell recognition and lysis, direct cytotoxic effect by granzyme B secretion.[34] Previous studies have demonstrated that high stromal infiltration of B cells assessed by multiplexed quantitative immuno-fluorescence or by IHC was independently and significantly correlated with a high pCR rate in early breast cancer.[9,10] Others have found out that the high expression of B cell-related transcripts was associated with a favorable survival in early breast cancer.[35,36]

Conclusion

Our study demonstrated that the presence of stromal TILs and TILs subsets ($CD3^+$, $CD4^+$, $CD8^+$ and $CD20^+$ TILs) was significantly associated with an increased pCR rate in early TNBC. The level of TILs and TILs subsets in tumor stroma could be clinically beneficial in identifying patients with early TNBC who may benefit from NAC. In the future, it will be worthwhile to both integrate the predictive information obtained from stromal TILs and TILs subpopulations in post-NAC residual tumor specimens, and to determine the changes induced by NAC on stromal TILs and TILs subsets.

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