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ORIGINAL ARTICLE

Prognostic Role of Immune Markers in Triple Negative Breast Carcinoma

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Abstract

Tumor immune microenvironment (TIME) is a significant prognostic parameter for triple negative breast carcinomas (TNBC) due to being a target for immunotherapeutic agents and its essential role during the cancer immunoediting process. In this study, CD8, FOXP3, CD163, PD-L1/SP142 and PD-L1/SP263 antibodies were examined in a sample of 51 TNBC cases. Patients who received neoadjuvant therapy were excluded. CD8, FOXP3 and CD163 antibodies were evaluated separately in intratumoral area (ITA) and tumor stroma (TS). PD-L1 status was also examined in tumor cells (TC) and immune cells (IC) using both SP142 and SP263 antibodies. In multivariate Cox regressions, the only antibody that was found to be significantly associated with survival was SP142. SP142-positivity in TC and IC was related to increased overall survival. Higher CD163 expression in ITA and SP263-positivity in IC were associated with younger age. Lymphatic/angioinvasion was more frequent in cases with negative/low CD8 and FOXP3 expressions. Moreover, metastatic axillary lymph node(s) was associated with negative/low FOXP3 expression in TS. CD8, FOXP3, CD163, SP142 and SP263 expressions were positively correlated with each other, except a mild discordance caused by CD163 in ITA. Although PD-L1 status with both SP142 and SP263 antibodies were concordant in the majority of cases, 33.3% and 13.7% of the cases showed SP142-negative/SP263-positive pattern in TC and IC respectively. In conclusion, we suggest that composition, density and localization of the immune cells and the check point molecules are important prognostic parameters in TNBC. Immunohistochemistry can be used as an accessible and less expensive tool to demonstrate TIME.

Keywords Triple negative breast cancer · Tumor infiltrating lymphocytes · Tumor associated macrophages · PD-L1 · Fork head box protein 3 · Immunohistochemistry

Introduction

Breast carcinoma (BC) is the most common cancer and the leading cause of cancer related death among women [1]. A sub-group of BCs, which do not show estrogen receptor (ER) and progesterone receptor (PR) expression as well as human epidermal growth factor 2 (HER2) amplification, the so-called triple negative breast carcinoma (TNBC), accounts for %15–20 of all the BCs [2–5]. Although TNBC is a heterogeneous tumor family that consists of different BC sub-types, these tumors share many clinical and pathological features, such as younger age, family history, race, obesity, lower socioeconomic status, breast cancer gene (BRCA) mutation or dysfunction, larger tumor size, tumor necrosis, higher histological grade (HG), vascular invasion and poor prognosis in spite of a better response to neoadjuvant chemotherapy [4–9]. Considering aggressive clinical behavior and restricted therapeutic options, it has become ever more important to understand histopathological and molecular characteristics of the TNBC not only to make a better prediction of prognosis, but also to develop new tailored therapies.

Tumor immune microenvironment (TIME) is a relatively novel aspect for many types of human tumor, of which BC is one of the most commonly researched ones. Cancer

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immunoediting (CIE) is based on the interaction between immune cells (IC) and tumor cells (TC). IC has complex effects on TC, some of which are paradoxical, during the dynamic process of CIE. Due to its key role in all of the three phases (elimination, equilibrium, escape) of the CIE; TIME is a significant factor for tumorigenesis, tumor progression, response to therapeutics, and prognosis [10–12].

Tumor infiltrating lymphocytes (TIL), an essential component of TIME, has been proposed in the literature as an important parameter for BC and specific recommendations to evaluate TIL have been offered by International TILs Working Group (TIL-WG) [13]. Presence and/or density of TIL and density of different lymphocyte sub-types in the TIL have marked prognostic effects on BCs, particularly on TNBCs. In the majority of the TNBCs, TIL largely consists of cytotoxic T lymphocytes (CTL) that are characterized by CD8 expression [14]. On the other hand, regulatory T lymphocytes (RTL), which express forkhead box protein 3 (FOXP3), form a smaller but no less significant part of TIL [14, 15]. Another IC type in TIME is the tumor associated macrophages (TAM). TAM tend to show anti-inflammatory and pro-tumorigenic effects like M2 macrophages, which can be demonstrated by CD163 expression [16].

Programmed cell death 1 (PD-1), a member of CD28/CTLA4 receptor family, is an immune checkpoint protein. PD-1 activates the cytotoxic immune response when it is not bound with Programmed cell death ligand 1 (PD-L1). Although PD-L1 has a physiological role in the immunological tolerance, it is found to be expressed on TC and IC in the TIME of many types of human tumor, including TNBC [17–20]. This finding suggests that PD-L1 expression can be a significant escape mechanism for tumor, giving rise to the studies on immune checkpoint modulatory agents, such as PD-L1 inhibitors. After the results of IMpassion130 trial, a PD-L1 inhibitor agent -atezolizumab- has been recently approved by Food and Drug Administration (FDA) for patients with locally advanced or metastatic TNBC that shows 1% or more PD-L1 expression in ICs, in combination with nab-paclitaxel therapy [21, 22].

Aim of this study is to evaluate CD8, FOXP3, CD163 and PD-L1 expressions in TNBC and to see their association with the histopathological and clinical parameters as well as the prognosis.

Materials and Methods

Patients

Resection specimens of primary BCs diagnosed between January 2012 and December 2017 at Marmara University Hospital, Istanbul were retrospectively examined. Specimen type, patients' age and sex, histopathological parameters, proliferation index, hormone receptor and HER2 status were extracted from original pathology reports. Patients who received neoadjuvant therapy, male BCs, tru-cut biopsy specimens, and non-triple negative BCs were not included. Negativity of ER and PR were defined as <1% TC displaying nuclear staining, while negativity of HER2 was defined as no or incomplete and faint/barely perceptible membrane staining >10% of TC and/or HER2/chromosome17 ratio < 2.0 and average HER2 copy number < 4.0 signal/cell with dual-probe HER2 fluorescence in situ hybridization, according to American Society of Clinical Oncology - College of American Pathologists (ASCO-CAP) guidelines [23]. Finally, a total number of 51 resection specimens diagnosed as primary TNBC without neoadjuvant therapy were included. No additional selection criteria were applied. The clinical follow up data of all cases was gathered from medical records and personal correspondence with patients.

Histopathological Parameters

Tumor type, Nottingham HG, tumor size, nodal status and lymphatic/angio invasion (L/AI) were extracted from original pathology reports. According to recommendations of TIL-WG, an average percentage of stromal TIL density within the borders of invasive tumor were decided on Hematoxylin-Eosin (HE) stained slides [13]. ICs in crush artifacts, necrotic areas and/or around ductal carcinoma in situ (DCIS) were not considered. TILs were classified as negative/low (0–9%), intermediate (10–49%) and high (≥50%), using three grade scale [24, 25]. Different thresholds between 30% and 60% had been suggested to determine lymphocyte-predominant breast carcinoma (LPBC) [24, 26–29]. TIL-WG recommended that threshold for LPBC may vary between %50 and 60% [13]. Cases with 50% or more TIL were considered as LPBC in this study.

Immunohistochemistry (IHC)

CD8, FOXP3, CD163, PD-L1/SP142 and PD-L1/SP263 antibodies were included in this study. CD8, which is known as a basic IHC marker and frequently used in many organ tumors, is used to evaluate cytotoxic T-lymphocytes. Regulatory T-lymphocytes, which consists a lesser known component of TIME with the conflicting prognostic aspects reported in the literature, are evaluated with FOXP3 IHC. CD163 was performed to visualize the M2-macrophages. Since the number of studies that focused on tumor associated M2-macrophages in TNBC cases was very limited in the literature, CD163 IHC was included in this study. PD-L1/SP142 and PD-L1/SP263 antibodies were used to evaluate PD-L1, considering its immunotherapeutic and prognostic aspects as well as the need for examining the concordance between different PD-L1 clones.

Immunohistochemistry was performed on 3-μm-thick full-face sections of formalin-fixed and paraffin-embedded tissues using the Ventana Benchmark XT automated stainer (Ventana Medical System, Inc., Tucson, AZ, USA). A biotin-free HRP multimer based ready-to-use DAB detection kit (ultraView™ Universal DAB Detection Kit, Ventana Medical System, Inc., Tucson, AZ, USA) was employed. The following primary antibodies were used in this study: CD8 (C8/144B, monoclonal, mouse, Dako, ready-to-use), FOXP3 (EP340, rabbit monoclonal, Epitomics, 1:100), CD163 (EP324, monoclonal, rabbit, Epitomics, 1:100), PD-L1/SP142 (SP142, monoclonal, rabbit, Ventana, ready-to-use) and PD-L1/SP263 (SP263, monoclonal, rabbit, Ventana, ready-to-use). ER (6F11, monoclonal, rabbit, Leica Biosystems, 1:100), PR (16, monoclonal, rabbit, Leica Biosystems, 1:100), HER2 (EP1045Y, monoclonal, rabbit, Thermo Scientific™ Lab Vision™, 1:100) and Ki67 (SP6, monoclonal, rabbit, Biocare Medical, 1:100) had been performed during routine pathological examination.

CD8 and FOXP3 stains were separately evaluated for intratumoral area (ITA) and tumor stroma (TS). In accordance with the recommendations of TIL-WG, average percentage of expression was determined in ITA and TS, without focusing on hot spots. To classify the cytoplasmic and membranous CD8 expression, same three grade scale as with the TILs was used. However, the three grade scale was not feasible for FOXP3 expression because of low expression levels and narrow range of the observed values. Nuclear FOXP3 expressions were dichotomized into negative/low and high categories using the median values of 1% for ITA and 2% for TS as thresholds [30]. CD163 stain was evaluated by the previously described quantitative hotspot method [31, 32]. After defining hotspot areas at scanning magnification, TAMs with cytoplasmic CD163 expression were counted in 5 HPFs separately for ITA and TS and the average counts per HPF were recorded. TAM counts per HPF were classified using a two-tiered scale according to median values (ITA: 7, TS: 22) [31, 32]. Membranous SP142 and SP263 expressions were individually evaluated in TCs and ICs. Considering that PD-L1 positivity had been defined as 1% or more SP142 expression in ICs for anti-PD-L1 treatment decision, we grouped SP142 and SP263 expressions as negative (expression <1%) and positive (expression ≥1%) for TC and IC [21]. For all five antibodies, expressions in crush artifacts, necrotic areas and/or DCIS were not included.

Statistical Analysis

Statistical analysis was performed using R-3.4.3 software. For the categorical variables, the Spearman's rank test was utilized to evaluate the correlations and the Chi Square test was used to test the differences. The difference between continuous variables was compared using the Mann Whitney U test. Overall survival (OS) was defined as the time period between the surgery and death or the latest observation date. Progression-free survival (PFS) was measured from the date of surgery to the date of local recurrence and/or distant metastasis. Survival probabilities were analyzed using the Kaplan-Meier estimates, Long-rank tests and Cox proportional hazard regressions. Statistical significance was considered as $p < 0.05$.

Results

Clinicopathological Characteristics

Median age was 49 (28–81). Forty (78.4%) cases were under 60 years old. Specimen type was lumpectomy in 34 (66.6%) and mastectomy in 17 (33.3%) cases. All cases were HG-3, thus statistical comparisons and/or survival analysis could not include HG. Categories of TIL densities are shown in Fig. 1. Eighteen (35.3%) cases had high density of TIL. Surgical margin was positive in one case (2%), which also showed local recurrence. Medical records and clinical follow up data were available for all patients, with a median follow up period of 48 months. All patients received anthracycline and taxane based adjuvant chemotherapy, which is the standard treatment of the center, while 35 patients (68.6%) also had adjuvant radiotherapy. Recurrence was seen in 10 (19.6%) cases; of which 5 had local recurrence, 2 had distant metastasis, and remaining 3 had relapsed in both. All patients received chemotherapy after they relapsed and anti-PD-L1 treatment was not given. Clinicopathological characteristics are summarized in Table 1.

Immunohistochemical Analysis

Table 2 presents the characteristics of IHC. Higher expressions of CD8, FOXP3 and CD163 were observed in TS as compared to ITA ($p < 0.01$). SP142 and SP263 expressions were also higher in IC than TC ($p < 0.01$). Expressions of CD8, FOXP3, CD163, SP142 and SP263 are shown in Fig. 2. There was a significant positive correlation between the five antibodies except that CD163 expression in ITA did not correlate with SP142 expression in TC and FOXP3 expression in TS ($p < 0.01$). In the majority of the cases, concordant PD-L1 positivity was reached with SP142 and SP263. However, 17 (33.3%) and 7 cases (13.7%) were PD-L1-negative with SP142 in TC and IC respectively, although they were positive with SP263. This discordance was significant ($p < 0.01$). None of the SP263-negative cases showed positivity with SP142. Median SP263 expression was higher compared to SP142 in TC (3% vs 0) and IC (15% vs 5%) Median Ki67 proliferation index was 60%.

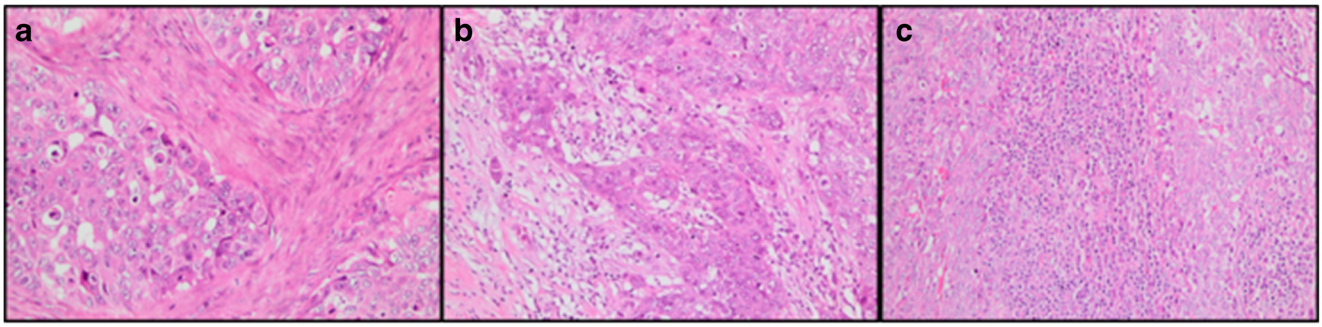

Fig. 1 TIL densities, Hematoxylin-Eosin, 200x. a) Negative/low b) Intermediate c) High

Comparison of IHC with the Histopathological Parameters

Patients with higher CD163 expression in TS and those with higher SP263 expression in IC were younger than the negative/low expression groups ($p < 0.05$ and $p = 0.05$, respectively). CD8 and FOXP3 expressions in both ITA and

Table 1 Clinicopathological characteristics

	Frequency *n, (%)*
Histological type	
NST	28 (54.9%)
LPBC	18 (35.3%)
Metaplastic	5 (9.8%)
Tumor size	
≤ 20 mm	18 (35.3%)
> 20 mm	33 (64.7%)
Nodal status	
pNX	4 (7.8%)
pN0	27 (39.2%)
pN1–2-3	20 (39.2%)
Lymphatic/angio invasion	
No	27 (52.9%)
Yes	24 (47.1%)
TIL	
Negative/Low	8 (15.7%)
Intermediate	25 (49.0%)
High	18 (35.3%)
Recurrence	
No	41 (80.4)
Yes	10 (19.6)
Exitus	
No	42 (82.4%)
Yes	9 (17.6%)

NST: No special type, LPBC: Lymphocyte-predominant breast carcinoma, TIL: tumor infiltrating lymphocytes

Table 2 Immunohistochemical characteristics

	Frequency *n, (%)*
CD8-ITA	
Negative/Low	28 (54.9%)
Intermediate	20 (39.2%)
High	3 (5.9%)
CD8-TS	
Negative/Low	5 (9.8%)
Intermediate	32 (62.7%)
High	14 (27.5%)
FOXP3-ITA	
Negative/Low	30 (58.8%)
High	21 (41.2%)
FOXP3-TS	
Negative/Low	26 (51%)
High	25 (49%)
CD163-ITA	
Negative/Low	26 (51%)
High	25 (49%)
CD163-TS	
Negative/Low	26 (51%)
High	25 (49%)
SP142-TC	
Negative	30 (58.8%)
Positive	21 (41.2%)
SP142-IC	
Negative	15 (29.4%)
Positive	36 (70.6%)
SP263-TC	
Negative	13 (25.5%)
Positive	38 (74.5%)
SP263-IC	
Negative	7 (13.7%)
Positive	44 (86.3%)

ITA: Intratumoral Area; TS: Tumor Stroma;
TC: Tumor Cell; IC: Immune Cell

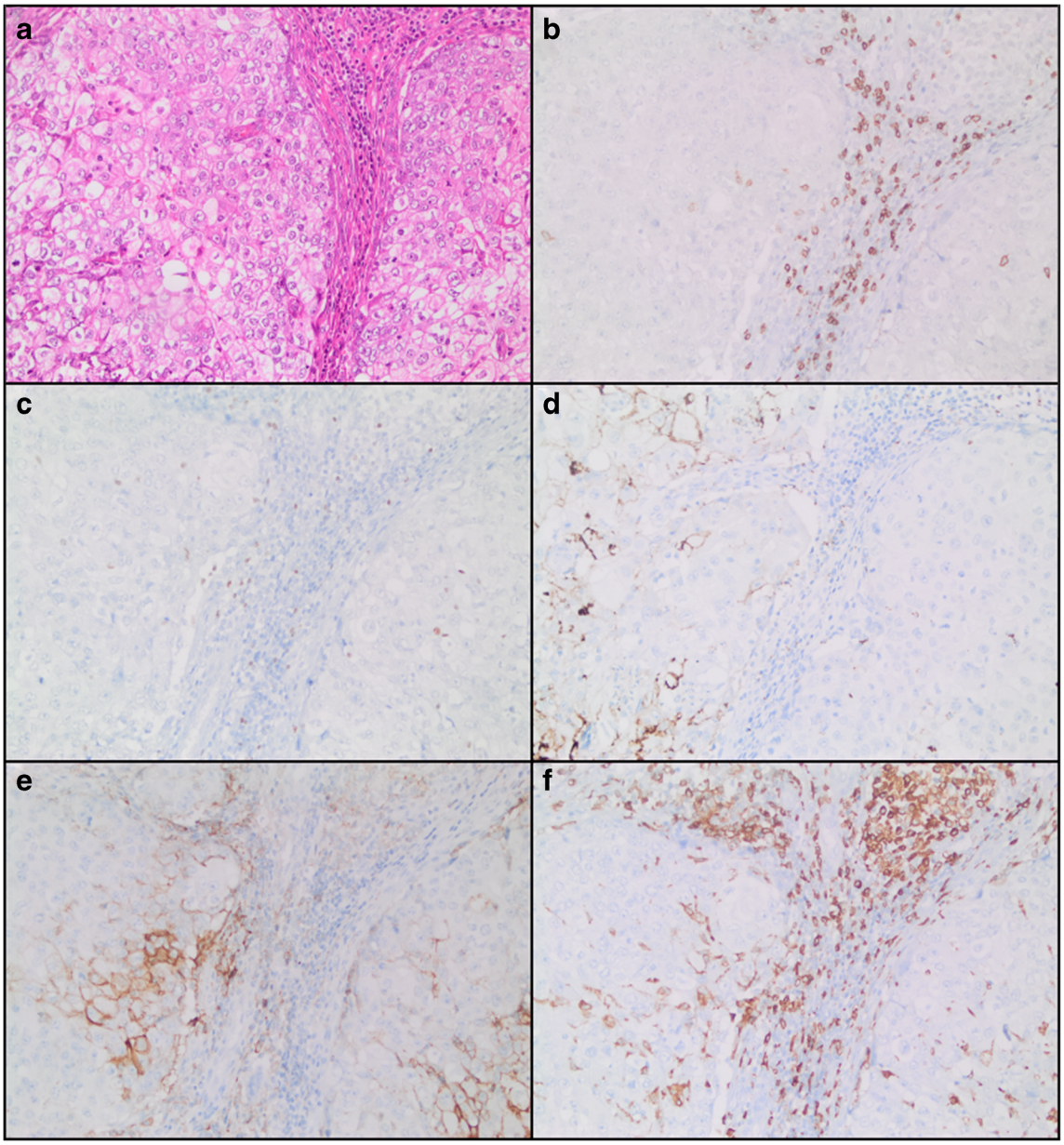

Fig. 2 Expressions of immunohistochemical markers, 200x. a) Hematoxylin-Eosin staining of the same area b) CD8 expression in intratumoral area and tumor stroma c) FOXP3 expression in intratumoral area and tumor stroma d) CD613 expression in intratumoral area and tumor stroma e) SP263 expression in tumor cells and immune cells f) SP142 expression in tumor cells and immune cells

TS, in addition to the SP142 expression in TC, were higher in LPBC cases ($p < 0.01$). CD8 expression in ITA and FOXP3 expression in ITA and TS were found to be lower in the cases with L/AI ($p < 0.05$). L/AI was negatively correlated with CD8 ($rho = -0.32$, $p = 0.01$) and FOXP3 ($rho = -0.39$, $p < 0.01$) expressions in ITA, as well as the FOXP3 expression in TS ($rho = -0.45$, $p < 0.01$). Additionally, lymph node involvement (pN1–2-3) was more frequent in the cases showing no/low FOXP3 expression in TS ($p = 0.05$). TIL evaluated on HE slides was positively associated with SP142 ($rho =$ 0.51(TC), 0.41(IC)), SP263 ($rho = 0.48$(TC), 0.33(IC)), CD8 ($rho = 0.45$(ITA), 0.78(TS)) and FOXP3 ($rho = 0.34$(ITA), 0.40(TS)) ($p < 0.01$). However, it was not related with CD163 expressions. Mean Ki67 index was higher in the high FOXP3 expression group in ITA compared to the negative/low group ($p < 0.05$).

Survival Analysis and Correlations

Five-year PFS and OS rates were 74.7% and 75.6% respectively. Median PFS was 19 and OS was 27 months. In Kaplan-Meier analyses, the only clinical and histopathological parameters that had a statistically significant relationship with OS were age and nodal status ($p = 0.01$ and $p = 0.05$). Older age

and positive lymph node(s) were found to be negative predictors of OS. No statistically significant association was found between clinical and histopathological parameters and PFS ($p > 0.05$).

Higher FOXP3 levels in ITA were associated with longer OS, while higher CD163 levels in TS were related with both longer OS and PFS ($p < 0.05$). Although higher expression levels showed a trend toward a better probability of OS and PFS, none of the other IHCs showed a statistically significant relation with survival. Kaplan Meier graphs for OS and PFS are shown in Figs. **3** and **4**. Age, tumor size, nodal status and L/AI status were defined as control variables in multivariate analysis. Controlling for a number of clinical and histopathological parameters, only SP142 expression significantly and substantially increases the probability of overall survival in TC (HR = 0.21 [0.06–0.68]) and IC (HR = 0.13 [0.03–0.47]). SP142 expression in TC and IC had a significant positive prognostic effect on OS in multivariate Cox regression models (Table 3). On the other hand, none of the IHCs showed significant association with PFS in the multivariate analysis.

In addition to the survival analysis, we searched for the correlations of IHCs with exitus and recurrence status. Exitus was found to be reversely-correlated with high FOXP3 expression in ITA ($rho = -0.28$, $p < 0.05$), as well as SP142 ($rho = -0.26$, $p = 0.06$) and SP263 ($rho = -0.26$, $p = 0.06$) expression in IC. Moreover, recurrence was less frequent in the cases which were SP263-positive in IC ($rho = -0.29$, $p < 0.05$) and had higher CD163 expression in TS ($rho = -0.32$, $p < 0.05$), respectively.

Discussion

TNBC is associated with a worse prognosis than other types of BC due to both its aggressive behavior and limited therapeutic options [33, 34]. Nevertheless, not all TNBC cases present with equally bad prognosis. Classification of TNBC cases is essential for predicting the prognosis and deciding the type of therapeutic agents to be included in the treatment, particularly the immunotherapeutics. There are several molecular typologies in the literature, however, more accessible and less expensive markers to be used in routine pathology practice are still needed [35–41].

TIME is a dynamic system, with ICs in motion and the release of various cytokines, shaped by the interactions between TC and immune system. In this study, we evaluated CTL, RTL and TAM populations and PD-L1 (SP142 and SP263) expressions in 51 TNBC cases without the history of neoadjuvant therapy to understand TIME and its prognostic role. TILs have been the most commonly researched component of TIME. TIL-WG published specific recommendations about how to evaluate TIL in BCs [13]. Although, these recommendations suggest that an evaluation of stromal TIL in HE slides is sufficient for routine practice, they encourage using IHC and considering TILs in both ITA and TS for research purposes. In a smiler way, although the requirement for applying atezolizumab treatment is defined as positive staining of SP142 antibody in IC, in the literature, there are numerous studies that investigated various PD-L1 antibodies in IC and/or TC. This study contributes to that body of knowledge by examining CD8, FOXP3, and CD163 in ITA and TS as well as SP142 and SP263 in TC and IC in addition to the stromal TILs in HE slides.

LPBC, which is indicated by higher levels of stromal TIL, is thought to be correlated with Lehmann's immunomodulatory molecular subgroup [41]. Higher stromal TIL level is accepted as a good prognostic factor for TNBC due to its association with better response to neoadjuvant therapy and it is well known that achieving pathologic complete response is associated with longer survival [16, 28, 29, 42, 43]. Neither the stromal TIL levels nor the LPBC subtypes were associated with survival and/or other prognostic parameters in this study. An important proportion of TIL is formed by CTLs. Higher CTL levels in ITA and/or TS had positive prognostic role in triple-negative or hormone-negative BCs [44–48], but neutral/negative prognostic role in hormone-positive BCs [45–47]. CTL expression was also found to be correlated with younger age (ITA and TS) and higher HG (TS) [46, 49]. In this study, CTL was not associated with any of the prognostic parameters or survival either in ITA or TS, except for a negative correlation between CTL in ITA and L/AI. The small study population might be an explanation as to why a statistically significant association between CTL and survival was not observed in this study.

The important and sometimes paradoxical effects of RTLs on prognosis have been recognized in recent years. According to Ladoire et al., RTLs had adverse prognostic effects in non-infected TIME (e.g. in BC), whereas they showed good prognostic effects in infected TIME (e.g. in colorectal adenocarcinoma) [15]. Considering the heterogeneity of the BC family, it is not surprising to see that paradoxical effects of RTLs have been reported in BCs. Focusing on triple-negative or hormone-negative populations, there are conflicting data about the effect of higher FOXP3 levels in TS on survival outcome in the literature. Higher FOXP3 levels in TS were found to be associated with both decreased [16, 50–52] and increased [47, 53–55] survival, in addition to the studies that did not find any relationship between RTLs and survival [56, 57]. RTLs in TS were also found to be correlated with younger age, higher HG, larger tumor size and L/AI in different studies [50, 52–54, 57]. We found that higher RTL level in ITA was a positive predictive marker for OS in the univariate analysis, but not in the multivariate analysis. Additionally, in this study, higher levels of RTL were related with LPBC subtype (ITA and TS) and higher proliferation index (ITA), yet it was

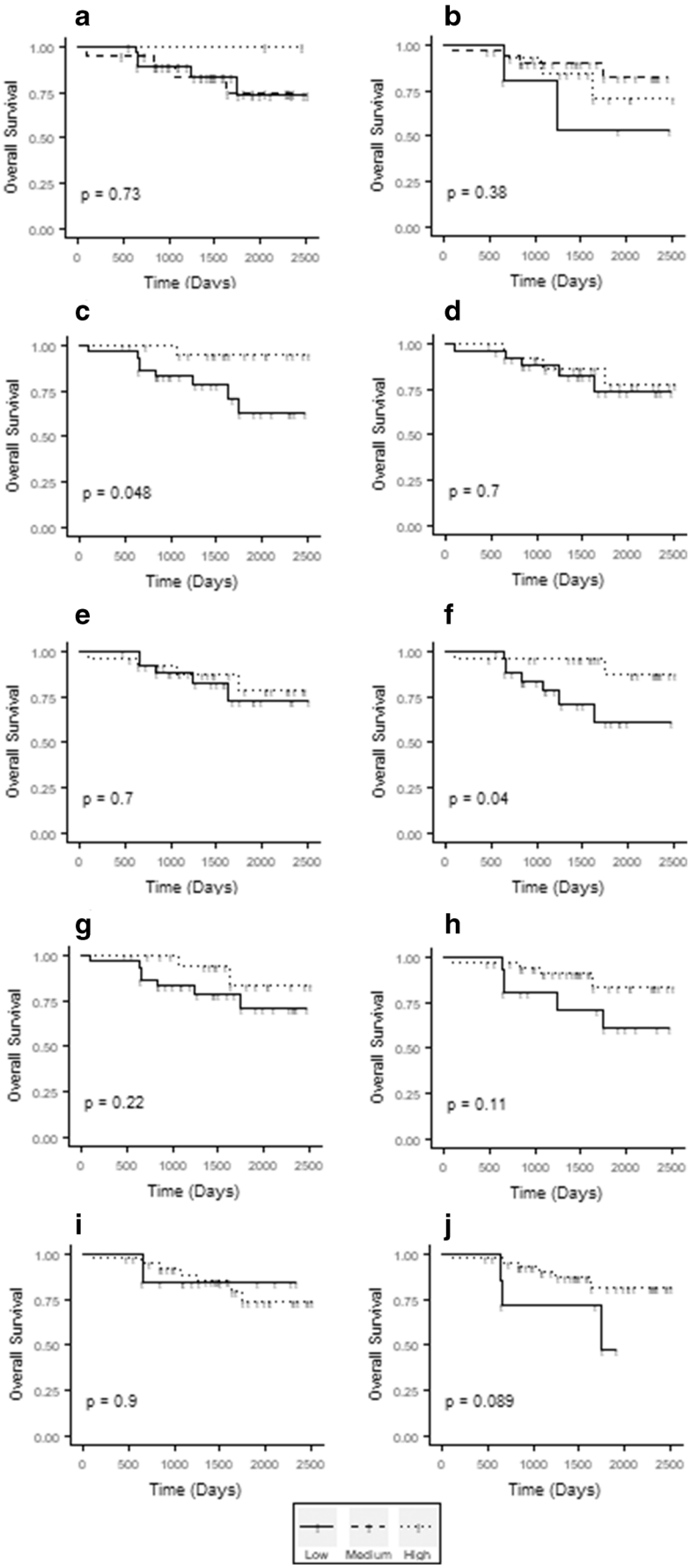

Fig. 3 Kaplan Meier graphs for overall survival a) Intratumoral CD8 expression b) Stromal CD8 expression c) Intratumoral FOXP3 expression d) Stromal FOXP3 expression e) Intratumoral CD163 expression f) Stromal CD163 expression g) SP142 expression in tumor cells h) SP142 expression in immune cells i) SP263 expression in tumor cells j) SP263 expression in immune cells

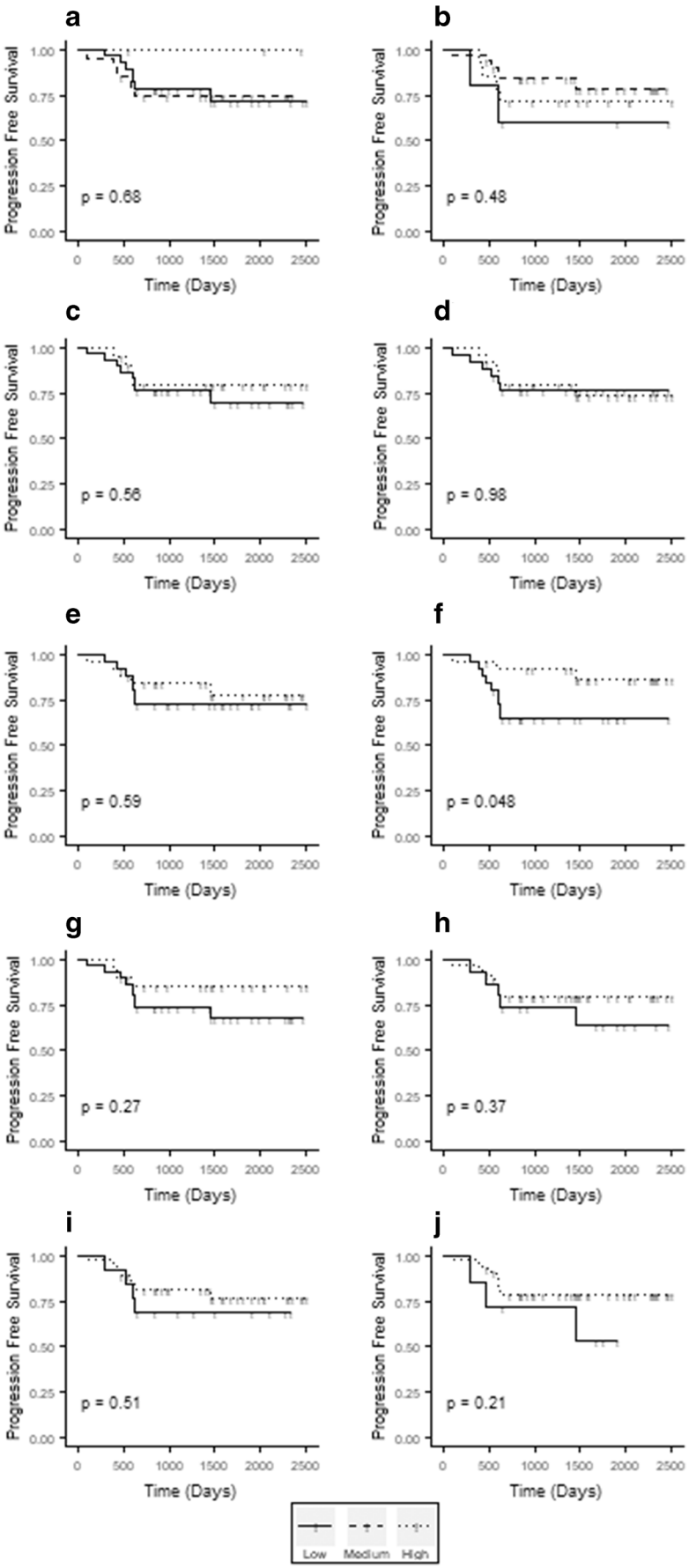

Fig. 4 Kaplan Meier graphs for progression-free survival a) Intratumoral CD8 expression b) Stromal CD8 expression c) Intratumoral FOXP3 expression d) Stromal FOXP3 expression e) Intratumoral CD163 expression f) Stromal CD163 expression g) SP142 expression in tumor cells h) SP142 expression in immune cells i) SP263 expression in tumor cells j) SP263 expression in immune cells

Table 3 Multivariate Cox Regression Models of Overall Survival

Variable	**Model 1**			**Model 2**		
	HR	***95% CI***	***p value***	***HR***	***95% CI***	***p value***
Age	11.55	1.80–74.05	**0.009**	16.10	2.76–93.66	**0.001**
pT	2.79	0.32–23.82	0.34	1.04	0.85–12.64	0.97
pN	9.83	1.24–77.87	**0.03**	16.87	1.75–161.75	**0.01**
L/AI	0.72	0.09–5.50	0.75	1.02	0.13–7.94	0.98
SP142 TC	0.21	0.06–0.068	**0.009**	–	–	–
SP142 IC	–	–	–	0.12	0.03–0.47	**0.002**

HR: Hazard-ratio; CI: Confidence intervals; L/AI: Lymphatic/Angio Invasion; TC: Tumor Cell; IC: Immune Cell

p-values are computed using Huber-White robust standard errors

reversely correlated with L/AI (ITA and TS) and lymph node metastasis (TS). Several factors could explain the positive prognostic effects of RTL in this study and other studies with similar results in the literature: the correlation between RTL and the other components of immune-rich microenvironment (e.g. TIL, CTL, TAM and PD-L1), association with younger age and LPBC subtype, potential role in response to systemic therapy, and the previously reported paradoxical biological behaviors in different tumors with different TIME. Few studies that utilized different FOXP3 clones described expression in BC cells [52, 58, 59], however, we observed FOXP3 expression only for lymphocytes.

TAM is a relatively lesser-known cellular component of TIME. T lymphocytes prompt the macrophage polarization (M1 or M2) via cellular cross-talking mechanisms [60]. TAMs mainly consist of M2 macrophages, which were found to be related with epithelial-mesenchymal transition, hyaluronan-rich composition of extracellular matrix and metastasis [31, 61]. Higher TAM levels in TS is associated with younger age, higher HG, larger tumor size, L/AI, triple/hormone-negativity and decreased survival in BCs [62, 63]. Adams et al. found that higher CD163 expression in TS was correlated with higher FOXP3 and PD-L1 expressions and a decreased OS probability in TNBC. In another study, Yang et al. reported that higher levels of CD163 were associated with higher HG, larger tumor size, and decreased OS and PFS in basal-like BCs [32]. The number of BC studies that focuses on TAM and uses a M2-spesific marker (e.g. CD163) is very limited. Moreover, only few studies examining M2 macrophages in TNBC group are present in the literature. In this study, higher M2-macrophage count in TS was associated with increased OS and PFS in univariate analysis, though it was not confirmed in multivariate analysis. Expression of CD163 was found in correlation with CD8, FOXP3, SP142 and SP263 expressions and younger age; thus, we regarded the positive prognostic effect of CD163 on survival as confounding, whilst real prognostic value of CD163 on survival still needs to be studied.

The effects of PD-L1 have become widely researched for many of the human tumors in recent years. This popularity is not only about the prognostic role of PD-L1, but also –maybe more importantly- about the use of immunotherapeutic agents in PD-L1 positive cases. Although PD-L1 is a protumorigenic molecule, which facilitates tumoral escape from the host's immune system, and anti-PD-L1 therapy has been accepted as a very powerful ally for the management of many of the human tumors including TNBC, it would not be appropriate to summarize the complex role of PD-L1 in prognosis as being the doomsayer, since recent studies has reported some good prognostic features in the PD-L1 positive cases [18, 20, 22]. An interferon-gamma-induced PD-L1 expression on tumor cells, which was driven by T cells in TIME, the so-called adaptive resistance, has been described [64, 65]. This finding might be an explanation for the frequent PD-L1 positivity in the tumors with higher TIL level, in concordance with our results. In addition to a TIL-rich microenvironment, PD-L1 expression was associated with a better response to (neo)adjuvant therapy and increased survival in BC, especially in TNBC [19, 55, 66–71]. However, there are several studies that reported decreased or stable survival in case of PD-L1 positivity [16, 71–74]. In this study, the only IHC that was statistically significantly related to OS in multivariate analysis was SP142. SP142-positivity in TC and IC was related to prolonged OS. We observed concordance between SP142 and SP263 in the majority of the cases. However, 33.3% and 13.7% of the cases showed SP142-negative/SP263-positive expression pattern in TC and IC, respectively. Correlation between different PD-L1 antibodies as well as discordantly lower expression of SP142 compared with the other antibodies have been reported in the literature, similar with our findings [75–78].

Co-existence of PD-L1, FOXP3 and/or CD8 expressions was observed in few studies in the literature [55, 70, 72, 79]. A positive correlation between each of the five antibodies in both compartments was also found in this study, with the exception of CD163 expression in ITA. Additionally, it is worth to mention that expression in TS/IC was significantly higher compared to ITA/TC for all of the IHCs in all cases.

In this study, we examined TIME in a population of TNBC cases with no history of neoadjuvant therapy and detailed clinical follow up. To our knowledge, this is the first time that CTLs, RTLs, TAMs and PD-L1 status (SP142 and SP263) were examined in tumoral and stromal compartments in a single study. According to our results, immune-rich microenvironment is a positive prognostic marker in TNBCs. Types, density and localizations of immune cells and check point molecules are important parameters while examining the TIME. A noteworthy finding is that some of these immune cells and molecules, like RTLs and PD-L1, may paradoxically be associated with good prognosis. We found that among the five antibodies, only SP142 expression in IC significantly

associated with increased overall survival in multivariate analysis. This can mainly be attributed to their co-existence with and induction by an immune-rich microenvironment as well as the positive impact they have on the response to (neo)adjuvant therapy. Since the study was based on a specific BC subtype and the cases who received neoadjuvant therapy were excluded, the sample size was limited. However, despite the small sample size, it was important and worthwhile to evaluate TIME in the TNBC cases without neo-adjuvant therapy; since the TNBC is one of the most immune-rich breast cancer subtypes with immune-targeted therapeutic options and it is known that the neo-adjuvant therapy affects the presence, diversity and density of the cellular and molecular components of TIME. Further studies on larger samples are still needed to assess the TIME of TNBC and its effects on prognosis.

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Conceptualization: Hulya Sahin Ozkan, Handan Kaya.
Methodology: Hulya Sahin Ozkan.
Formal analysis and investigation: Hulya Sahin Ozkan.
Data collection - histopathological and immunohistochemical: Hulya Sahin Ozkan.
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Availability of Data and Material The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

Conflicts of Interest/Competing Interests The authors declare that they have no conflicts of interest and/or competing interests.

Ethics Approval The study protocols were approved by the Ethical Board of the Marmara University Medical School, Istanbul, Turkey (Protocol number: 09.2017.665).

Consent to Participate N/A

Consent for Publication N/A

Code Availability R code of the statistical analyses is available from the corresponding author on reasonable request.

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