Original Study

Tumor-Infiltrating Lymphocytes (TIL), Tertiary Lymphoid Structures (TLS), and Expression of PD-1, TIM-3, LAG-3 on TIL in Invasive and In Situ Ductal Breast Carcinomas and Their Relationship with Prognostic Factors

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Abstract

We wanted to evaluate the expression of immune checkpoint receptors in pathology samples to support research on whether the immune response against breast cancer can be used for treatment. For this purpose, we studied 312 invasive and 68 in situ breast cancers. We found that PD-1 and stromal TIL were associated with survival. Determination of PD-1 expression on stromal TIL can provide information about whether the patient can benefit from immunotherapy.

Introduction: Immunotherapy has been determined as an important choice in breast carcinomas, especially in tumors with markedly inflammatory response. About this promising subject, tumor-infiltrating lymphocytes (TIL) and the expression of immune control point receptors on TIL have gained importance. **Materials and Methods:** In this study, stromal TIL and tertiary lymphoid structures (TLS) were determined in tumor tissues of 312 invasive and 68 in situ breast cancer patients. Expression rates of PD-1, LAG-3, and TIM-3 on intratumoral and stromal TIL were immunohistochemically evaluated. **Results:** In invasive breast carcinomas, stromal TIL was found to be significantly associated with lymph node metastasis, HR and HER2 expression, and basal-like phenotype, as the presence of TLS with neoadjuvant therapy, recurrence, death, and expression of HR and HER2. PD-1, LAG-3, and TIM-3 expressions were found to be associated with HR and HER2 status, stromal TIL rates, and TLS. In multivariate analysis, high stromal TIL and PD-1 expression in intratumoral TIL were found to be independent prognostic factors in terms of overall survival and disease-free survival. **Conclusion:** Evaluation of TIL and immune control point receptor expressions in breast cancer is particularly important in terms of planning the therapeutic approaches based on immunotherapy protocols.

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Keywords: Immune checkpoint molecules, Tumor immunology, Immunotherapy, Breast cancer treatment, Breast cancer prognosis

Introduction

Breast cancer is the most common cancer seen in women. The prevalence of breast cancer is increasing all over the world, and despite all kinds of treatment methods, it is reported that this cancer causes an average of 627,000 deaths per year.[1] In addition to surgical treatment, the treatment of breast cancer is shaped according to the hormone receptor status [HR (estrogen and progesterone receptors-ER and PR)], c-erbB-2 (HER2) expression, and Ki67 proliferation index of the tumor. As it is known, ER modulators such as tamoxifen are administered in patients with positive HR; monoclonal antibodies developed against the HER2 oncogene, such as trastuzumab, in patients with score 3 positive c-erbB-2; and, cytotoxic chemotherapeutic agents (such as paclitaxel and doxorubicin) in patients with negative HR and HER2 [triple (-)] tumors. These treatments shaped by the immunohistochemical staining profile may not always produce the desired results due to the molecular subtype difference of the tumor, additional diseases of the patient, and the side effects of the drugs used.[2] However, in recent years, immunotherapy has become a promising treatment option for especially triple (-) breast

Abbreviations: TIL, Tumor-Infiltrating Lymphocytes; TLS, Tertiary Lymphoid Structures.

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carcinoma, accompanied by an inflammatory response. The studies have been started on the use of immunotherapeutic agents in combination or as a single agent in order to avoid the negative effects of chemotherapy and increase the effectiveness of treatment, especially in triple (-) breast cancer patients.[3-5]

Immunotherapy have been studied by oncologists and immunologists for many years. The effects of chemotherapy and radiotherapy on the death of proliferating cells and arrest of cell division affect not only tumor cells but also normal cells. This situation causes an increase in mortality and morbidity in breast cancer patients. Therefore, research and development of more specific treatment methods such as immunotherapy have gained speed.[6] The most recent targets in immunotherapy are the immune control point receptors, which are programmed cell death protein-1 (PD-1) and its ligand (PD-L1), T-lymphocyte associated antigen-4 (CTLA-4), lymphocyte activation gene (LAG-3), T-cell immunoglobulin, and mucin domain-containing molecule 3 (TIM-3). These receptors are highly expressed in regulatory T-cells and have a negative role in T-cell proliferation and activation.[7]

The efficacy of immune checkpoint molecules is being investigated in tumors involving immune cell infiltration. The first parameter to be determined is the density of tumor-infiltrating lymphocytes (TIL) within the tumor, and then, the expression of inhibitor receptors on these lymphocytes. It has been understood that these receptors, which are used as targets in treatment, are closely related to prognosis. It has been reported that the density of TIL in breast cancer is found to be related to both the neoadjuvant chemotherapy response and the prognosis of patients who will receive adjuvant chemotherapy. Also, TIL are thought to be effective in transforming an in situ tumor into an invasive tumor.[8,9]

Another subject that has recently been investigated within an invasive tumor is the relationship between tertiary lymphoid structures (TLS) with prognosis. TLS are ectopic lymphoid formations similar to secondary lymphoid organs.[10] Previously, the presence of TLS in different cancers (lung cancer, colon cancer, and melanoma) has been investigated in terms of prognosis.[11-13] Studies also report that these structures have a prognostic significance in breast cancer.[14,15]

In this study on invasive and in situ ductal breast carcinomas, we aimed to determine the relationship between the density of TIL with ER, PR, and HER2 expressions of tumor cells as well as the expression profiles of new generation immune checkpoint molecules (PD-1, LAG-3, and TIM-3) and to associate the obtained data with prognostic factors and survival of breast cancer patients.

Materials and Methods

Patient Selection

A total of 380 breast cancer cases were included in this study whom 312 were diagnosed as invasive ductal carcinoma between the years 2010 to 2018 and 68 as ductal carcinoma in situ (DCIS) between 2010 and 2015 in the Department of Pathology, Faculty of Medicine, Gazi University, Ankara, TURKEY. The pathological materials consisting of hematoxylin and eosin (H&E) as well as immunohistochemically stained slides representing the tumor area obtained from total or partial mastectomy specimens were reassessed by 2 pathologists together.

The pathology reports and medical files of the selected cases were screened for histopathological and clinical data, respectively. Patients with the diagnosis of invasive ductal carcinoma including those with medullary pattern were grouped according to their histological grades and ER-PR-HER2 expression status. Patients with DCIS were selected among those without any accompanying invasive carcinoma component (except microinvasion) and those previously diagnosed as invasive carcinoma via excisional biopsy or mastectomy was excluded from the study. The information including prognostic factors such as tumor size, histological grade, ER-PR-HER2 expression, Ki67 proliferation index, and metastatic lymph node status of the selected cases were obtained from pathology reports; whereas distant metastasis status, pathological stage, neoadjuvant treatment, recurrence, disease-free survival, and overall survival status from medical files via hospital information management system.

The study was approved by the Gazi University Faculty of Medicine Clinical Research Ethics Committee in terms of ethics on April 29, 2019, with the meeting number 04.

Histopathological Evaluation

TIL evaluation was done retrospectively on H&E slides via light microscopy. While evaluating TIL, the guides of International Immuno-Oncology Biomarker Working Group on Breast Cancer and International TILs Working Group were considered.[8,16] TIL can be evaluated separately in the stromal, intratumoral and, peritumoral areas in breast cancer. Stromal TIL is defined as the inflammatory cells among tumor groups, while intratumoral TIL as the inflammatory cells within the tumor islands. However, the evaluation of TIL in the stromal areas is recommended due to its repeatability and ease.[16] Therefore, in our study, we preferred evaluating stromal TIL.

For the evaluation of TIL in invasive carcinoma cases, the best tissue samples representing the tumor were chosen according to the presence of stromal TIL density, lymphoid aggregates/TLS, and peritumoral parenchymal inflammation. Besides these features, presence of accompanying DCIS, with or without TIL, was noted. While making this choice, the quality of tissue processing related to the tumor area, which was considered to be very important, was also taken into consideration. The necrotic areas and areas with dense central hyalinization within the tumor were not evaluated. In tumor samples having a heterogeneous TIL density, whole-mount sections were examined at 200x magnification and the mean values were calculated.[8,16] On the other hand, for the evaluation of DCIS, not only the density of stromal TIL was determined but morphological patterns (ie, micropapillary, cribriform, solid, and papillary), presence of comedonecrosis, apoptosis, calcification, and healing phenomenon were noted, as well.

The density of stromal TIL is classified as mild (1%-10%), moderate (11%-49%), and high ($\geq$ 50%) both in invasive and *in situ* ductal carcinomas.

Immunohistochemical Evaluation and Scoring

Cases with clinical follow-up information and density of stromal TIL of more than 3% were included in the immunohistochemical study (98 of invasive, 23 of in situ ductal carcinoma cases). The tumor samples were all formalin-fixed and paraffin-embedded

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tissues and approximately 4-micron thick sections were prepared from each tissue block for immunohistochemical examination. CD3, PD-1, LAG-3, and TIM-3 were stained immunohistochemically by using a closed automated slide stainer (Ventana Benchmark XT). Tonsil tissue was used as a positive control for all antibodies. The clones and concentrations of these antibodies were as follows: CD3 (clone 2GV6, ready to use), PD-1 (clone NAT105, ready to use), LAG-3 (clone D2G40, dilution 1:200) and TIM-3 (clone D5D5R, dilution 1:400).

The density of intratumoral TIL was evaluated by determining the percentage of the tumor area occupied by CD3 positive T lymphocytes within tumor islands. The density of intratumoral TIL is classified similar to stromal TIL evaluation (ie, mild, medium, and high).

Cytoplasmic and/or membranous PD-1, LAG-3 and TIM-3 staining in lymphocytes were considered specific for the immune reaction pattern. Stained lymphocytes were scored separately for intratumoral and stromal TIL. The ratio of stained lymphocytes to total lymphocytes was evaluated by examining 3 independent microscopic high-power fields (x400) and then expressed as a percentage value. According to this, less than 5% was considered as low expression, while 5% as high. Total lymphocyte count in the intratumoral area was determined by CD3 staining.

Statistical Analyses

The analysis of the data was done using IBM SPSS version 22. The relationship between TIL, TLS and, immune checkpoint molecules expression on TIL and clinical features was evaluated by chi-square test. The mean ± standard deviation for age was calculated and the mean age difference among the groups was evaluated with the One-Way Anova test. Kaplan-Meier method was used for the effect of clinicopathological characteristics of patients on survival and compared with the log-rank test. Univariate and multivariate Cox regression models were used to investigate independent prognostic factors. In all tests, $P \leq .05$ was considered statistically significant.

Results

Baseline Clinical Features

In our study, 312 invasive breast carcinoma cases aged between 26 and 84 years were reevaluated and further studied. The mean age was 52.2. Only 5 cases were male and the rest were female. The mean tumor diameter was 3.1 ± 2.3 cm. The median follow-up period of the cases was 68 months (min. 2 months, max. 123 months). The 5-year overall survival rate of these cases was found to be 80%, whereas the 10-year overall survival rate was 72%. We are able to access clinical follow-up information 169 out of 312 cases. With the data obtained, the median disease-free survival time was found to be 61 months (min. 6 months, max. 120 months). In patients with available clinical follow-up information, the 5-year and the 10-year disease-free survival rates were 72% and, 69%, respectively.

In terms of DCIS, the number of cases was 68. The cases were aged between 29 and 77, with a mean of 53.8. All cases were female. The mean tumor diameter was 2.1 ± 2.5 cm.

Stromal TIL Density: Relationship With Pathological Features and Prognosis

High stromal TIL density was significantly found to be associated with basal-like phenotype and medullary-like invasive ductal carcinomas as expected ($P < .001$). It was also observed in triple-negative breast carcinomas and HR (-)/HER2 (+) tumors ($P = .001$); and also in ER and PR negative tumors ($P < .001$ and $P = .004$, respectively). Among other histological parameters examined, the relationship between TIL density of the invasive tumor with TLS and with accompanying DCIS is also found to be significant ($P < .001$) (Table 1). It was also shown that, in terms of DCIS, those cases who were HR (-)/HER2 (+) and those with tumor grade 3 of 3 had higher stromal TIL density ($P < .001$ and $P = .022$, respectively). In DCIS, as in invasive carcinomas, TLS were also found to be associated with higher stromal TIL density ($P < .001$). This was particularly noticeable in DCIS that show a healing phenomenon ($P < .001$) (Supplementary Table 1).

The effect of TIL density on the overall survival of 312 cases diagnosed with invasive breast carcinoma was investigated. In univariate analyzes performed with the Kaplan-Meier method, a statistically significant relationship was determined between stromal TIL density and overall survival only in the HR (-)/HER2 (+) group ($P = .007$) (Figure 1). Overall survival was higher in cases with high stromal TIL density. In this group, there was no statistically significant relationship between the stromal TIL density and disease-free survival ($P = 0.064$). Nevertheless, disease-free survival tends to be lower in those cases with lower stromal TIL density. The effect of stromal TIL density on survival was investigated by univariate Cox regression test and a statistically significant association was found between overall survival and TIL (Hazard ratio (HazR) = 0.6, 95% confidence interval (CI) = 0.4-0.9, $P = .050$), and also between disease-free survival and TIL (HazR = 0.5, 95% CI = 0.3-0.9, $P = .042$) in invasive breast carcinoma cases. In the multivariate Cox regression test, it was determined that tumor stage, stromal TIL density, ER expression, and HER2 overexpression were each determined to be independent prognostic factors, in terms of overall survival. In addition, the stromal TIL density and ER expression have been shown to be independent prognostic factors in terms of disease-free survival (Table 2).

TLS: Relationship with Pathological Features and Prognosis

A statistically significant relationship was observed between TLS and neoadjuvant treatment, relapse and death ($P = .025$, $P = .043$, and $P = .009$, respectively). More TLS were observed in basal-like phenotype and medullary-like invasive ductal carcinomas ($P = .048$), as in triple (-) and HR (-)/HER2 (+) tumors ($P = .019$). ER and PR negativity were found to be associated with the presence of TLS ($P = .004$ and $p = 0.013$, respectively). A higher rate of TLS were observed in tumors with higher stromal TIL density ($P < .001$) (Supplementary Table 2).

In univariate analyzes performed with the Kaplan-Meier method, a statistically significant relationship was found between the presence of TLS and overall survival ($P = .023$) (Figure 1). Overall survival was higher with TLS, and there was no statistically significant relationship between the presence of TLS and disease-free

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Table 1 The Relationship Between sTIL Density With Clinicopathological and Histological Parameters in Invasive Breast Tumors

Parameters	Patients	Density of sTIL			
	n (%)	Mild (1%-10%)	Moderate (11%-49%)	High (≥50%)	*P*
Age at diagnosis	312	176	70	66	.919
Mean ± SD	52.2 ± 12.8	52.5 ± 13.4	51.7 ± 11.5	52.1 ± 12.6	
Death	312				.084
Present	69 (22.1)	47 (68.1)	11 (15.9)	11 (15.9)	
Absent	243 (77.9)	129 (53.1)	59 (24.3)	55 (22.6)	
Recurrence	169				.052
Present	46 (27.2)	34 (73.9)	8 (17.4)	4 (8.7)	
Absent	123 (72.8)	67 (54.5)	29 (23.6)	27 (22.0)	
Neoadjuvant therapy	169				.029
Present	35 (20.7)	25(71.4)	9 (25.7)	1 (2.9)	
Absent	134 (79.3)	76 (56.7)	28 (20.9)	30 (22.4)	
pT	300				.715
pT1	99 (33.0)	51 (51.5)	25 (25.3)	23 (23.2)	
pT2	163 (54.3)	96 (58.9)	34 (20.9)	33 (20.2)	
pT3	32 (10.7)	19 (59.4)	8 (25.0)	5 (15.6)	
pT4	6 (2.0)	4 (66.7)	0 (-)	2 (33.3)	
pN	306				.040
pN0	134 (43.8)	80 (59.7)	21 (15.7)	33 (24.6)	
pN1-2-3	172 (56.2)	93 (54.1)	47 (27.3)	32 (18.6)	
pM	305				.272
pM0	298 (97.7)	166 (55.7)	68 (22.8)	64 (21.5)	
pM1	7 (2.3)	6 (85.7)	0 (-)	1 (14.3)	
TNM stages	303				.356
I	63 (20.8)	32 (50.8)	14 (22.2)	17 (27.0)	
II	138 (45.5)	85 (61.6)	27 (19.6)	26 (18.8)	
III	95 (31.4)	48 (50.5)	26 (27.4)	21 (22.1)	
IV	7 (2.3)	6 (85.7)	0 (-)	1 (14.3)	
Histologic type	312				<.001
Invasive ductal carcinoma, NOS	263 (84.3)	155 (58.9)	64 (24.3)	44 (16.7)	
Invasive ductal carcinoma with basal-like phenotype	38 (12.2)	19 (50)	6 (15.8)	13 (34.2)	
Invasive ductal carcinoma with medullary pattern	8 (2.6)	0 (-)	0 (-)	8 (100)	
Invasive ductal carcinoma showing signet ring cell morphology	2 (0.6)	2 (100)	0 (-)	0 (-)	
Invasive ductal carcinoma with metaplastic features	1 (0.3)	0 (-)	0 (-)	1 (100)	
Receptor expression	307				.001
Triple (-)	97 (31.6)	48 (49.5)	22 (22.7)	27 (27.8)	
HR (-)/HER2 (+)	64 (20.8)	27 (42.2)	15 (23.4)	22 (34.4)	
HR (+)/HER2 (+)	74 (24.1)	51 (68.9)	17 (23)	6 (8.1)	
HR (+)/HER2 (-)	72 (23.5)	49 (68.1)	13 (18.1)	10 (13.9)	
Histologic grade	312				.676
1	3 (1.0)	3 (100)	0 (-)	0 (-)	
2	18 (5.8)	11 (61.1)	5 (27.8)	2 (11.1)	
3	291 (93.3)	162 (55.7)	65 (22.3)	64 (22.0)	
ER expression	307				<.001
Negative (0)	157 (51.1)	73 (46.5)	36 (22.9)	48 (30.6)	
Low (1%-10%)	15 (4.9)	8 (53.3)	3 (20.0)	4 (26.7)	
High (>10%)	135 (44.0)	93 (68.9)	29 (21.5)	13 (9.6)	

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Table 1 (*continued*)

Parameters	Patients	Density of sTIL			
	n (%)	Mild (1%-10%)	Moderate (11%-49%)	High (≥50%)	*P*
PR expression	299				.004
Negative (0)	166 (55.5)	80 (48.2)	38 (22.9)	48 (28.9)	
Low (1-20%)	53 (17.7)	38 (71.7)	8 (15.1)	7 (13.2)	
High (>20%)	80 (26.8)	50 (65.0)	18 (22.5)	10 (12.5)	
HER2 overexpression/amplification	307				.854
(IHC and ISH)					
Present	138 (45.0)	78 (56.5)	32 (23.2)	28 (20.3)	
Absent	169 (55.0)	97 (57.4)	35(20.7)	37 (21.9)	
Ki67 proliferative index	94				1.000
Low (1%-15%)	7 (7.4)	4 (57.1)	1 (14.3)	2 (28.6)	
High (> 15%)	87 (92.6)	43 (49.4)	19 (21.8)	25 (28.7)	
TLS	312				<.001
Present	37 (11.9)	6 (16.2)	10 (27.0)	21 (56.8)	
Absent	275 (88.1)	170 (61.8)	60 (21.8)	45 (16.4)	
Lymphoid aggregate	311				.004
Present	88 (28.3)	49 (55.7)	29 (33.0)	10 (11.4)	
Absent	223 (71.7)	127 (57)	41 (18.4)	55 (24.7)	
Intensity of inflammation in non-	244				.715
tumoral breast parenchyma					
Mild (1%-10%)	162 (66.4)	93 (57.4)	37 (22.8)	32 (19.8)	
Moderate (11%-49%)	50 (20.5)	25 (50.0)	12 (24.0)	13 (26.0)	
High (≥ 50%)	32 (13.1)	15 (46.9)	8 (25.0)	9 (28.1)	
Accompanying DCIS	312				.439
Present	132 (42.3)	80 (60.6)	27 (20.5)	25 (18.9)	
Absent	180 (57.7)	96 (53.3)	43 (23.9)	41 (22.8)	
TIL density in accompanying DCIS	132				<.001
Mild (1%-10%)	77 (58.3)	58 (75.3)	12 (15.6)	7 (9.1)	
Moderate (11%-49%)	22 (16.7)	11 (50.0)	9 (40.9)	2 (9.1)	
High (≥50%)	33 (25.0)	11 (33.3)	5 (15.2)	17 (51.5)	

Stil = Stromal tumor infiltrating lymphocytes; SD = Standard Deviation; IHC = Immunohistochemistry; ISH = In situ hybridization

survival (*P*= .062). However, it has been observed that patients with TLS tend to have higher disease-free survival. Overall survival analysis performed with univariate Cox regression test revealed higher overall survival in the presence of of TLS (HazR = 0.2, 95% CI 0.1-0.9, *P*= .038). No independent prognostic factor could be shown in multivariate analyzes (Table 2).

PD-1, LAG-3, and TIM-3 Expressions: Relationship With Pathological Features and Prognosis

Higher PD-1 expression was observed on intratumoral TIL in cases who did not receive neoadjuvant therapy, did not relapse, and did not die (*P*= .012, *P*= .001, and *P*< .001, respectively), as well as in cases who had higher stromal TIL density and had TLS (*P*< .001 and *P*= .001, respectively). However, no statistically significant relationship was found between other histopathological features and PD-1 expression on TIL. PD-1 expression on the stromal TIL was observed to be higher in cases having invasive ductal carcinomas in the medullary pattern, HR (-)/HER2 (+) immunoprofile, and high stromal TIL density (*P*= .037, *P*= .001, and *P*= .002, respectively). High stromal PD-1 expression was found to be statistically significant in HER2 (+) cases when analyzed considering only HER2 expression, regardless of HR status (*P*< .001) (Table 3).

In invasive ductal carcinomas with basal-like phenotype and medullary pattern, higher LAG-3 expression was observed in both intratumoral and stromal TIL (*P*= .001 and *P*= .015, respectively). High LAG-3 expression in intratumoral TIL was observed in cases with high density of stromal TIL and with TLS (*P*< .001 and *P*= .002, respectively), as well as high in stromal TIL (*P*< .001 and *P*= .035) (Table 4).

Considering TIM-3, it was seen that its expression was high on both intratumoral and stromal TIL in invasive ductal carcinomas with basal-like phenotype and medullary pattern (*P*= .001 and *P*= .007, respectively). In addition, in cases with high stromal TIL density and with TLS, higher TIM-3 expression was observed on intratumoral TIL (*P*< .001 and *P*< .001, respectively), as well as on stromal TIL (*P*< .001 and *P*= .013, respectively) (Table 5).

When DCIS cases were evaluated, no significant difference was found between PD-1 and/or LAG-3 expressions on intratumoral and/or stromal TIL and clinicopathological parameters. By the way, TIM-3 expression was not detected on intratumoral TIL at all. High

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Figure 1 Association of overall and disease-free survival with TIL, TLS, and PD-1 in invasive breast tumors.

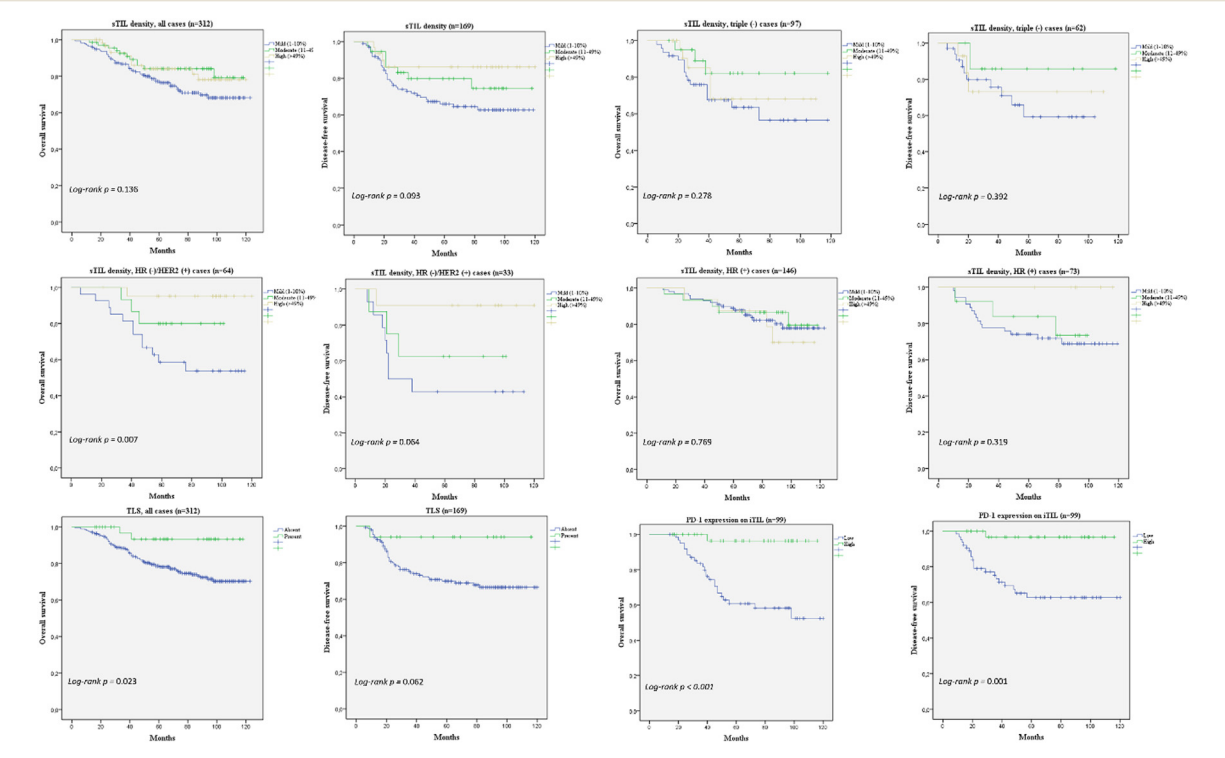

Table 2 Cox Regression Analysis for Overall Survival and Disease-Free Survival in Invasive Breast Tumors

	Univariate			Multivariate		
Overall Survival n = 312	**HazR**	**%95 CI**	***P***	**HazR**	**%95 CI**	***P***
Age at diagnosis (≥ 40 / < 40 years)	1.046	0.572-1.913	.883	-	-	-
Tumor size (> 2 / ≤ 2 cm)	2.356	1.260-4.404	.007	1.804	0.938-3.469	.077
Lymph node metastasis (present / absent)	2.672	1.526-4.679	.001	1.331	0.645-2.747	.439
Stage (III-IV / I-II)	3.720	2.280-6.069	<.001	3.513	1.832-6.736	<.001
Density of stromal TIL (moderate-high / mild)	0.602	0.363-0.999	.050	0.453	0.260-0.791	.005
TLS (present / absent)	0.226	0.055-0.923	.038	0.145	0.020-1.064	.058
ER expression (positive / negative)	0.532	0.327-0.864	.011	0.354	0.210-0.599	<.001
HER2 over expression (present/ absent)	0.620	0.381-1.010	.055	0.447	0.265-0.754	.003
Disease-free survival n = 169						
Age at diagnosis (≥ 40 / < 40 years)	0.707	0.366-1.367	.303	-	-	-
Tumor size (> 2 / ≤ 2 cm)	1.747	0.863-3.537	.121	1.466	0.691-3.113	.319
Lymph node metastasis (present / absent)	2.217	1.145-4.293	.018	1.760	0.771-4.017	.179
Stage (III-IV / I-II)	0.493	0.274-0.887	.018	1.611	0.764-3.400	.210
Density of stromal TIL (moderate-high / mild)	0.505	0.262-0.976	.042	0.392	0.189-0.813	.012
TLS (present / absent)	0.187	0.026-1.356	.097	0.263	0.035-1.985	.195
ER expression (positive / negative)	0.720	0.400-1.298	.275	0.524	0.276-0.996	.049
HER2 over expression (present/ absent)	1.041	0.583-1.858	.893	0.739	0.382-1.429	.368

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Table 3 The Relationship Between PD-1 Expression on iTIL and sTIL With Clinicopathological and Histological Parameters in Invasive Breast Tumors

		PD-1 Expression on iTIL, n (%)			PD-1 Expression on sTIL, n (%)		
	n (%)	Low (< 5%)	High (≥ 5%)	*P*	Low (<5%)	High (≥5%)	*P*
Age at diagnosis	99			.056			.040
< 40	16 (16.2)	7 (43.8)	9 (56.3)		4 (25.0)	12 (75.0)	
≥ 40	83 (83.8)	57 (68.7)	26 (31.3)		44 (53.0)	39 (47.0)	
pT	97			.536			.084
pT1	28 (28.9)	17 (60.7)	11 (39.3)		17 (60.7)	11 (39.3)	
pT2	58 (59.8)	40 (69)	18 (31)		29 (50.0)	29 (50.0)	
pT3	10 (10.3)	5 (50.0)	5 (50.0)		2 (20.0)	8 (80.0)	
pT4	1 (1.0)	1 (100)	0 (-)		0 (-)	1 (100)	
pN	98			.130			.092
pN0	39 (39.8)	27 (69.2)	12 (30.8)		24 (61.3)	15 (38.5)	
pN1	23 (23.5)	14 (60.9)	9 (39.1)		10 (43.5)	13 (56.5)	
pN2	16 (16.3)	7 (43.8)	9 (56.3)		4 (25.0)	12 (75.0)	
pN3	20 (20.4)	16 (80.0)	4 (20.0)		10 (50.0)	10 (50.0)	
pM	98			1.000			.490
pM0	97 (99)	63 (64.9)	34 (35.1)		47 (48.5)	50 (51.5)	
pM1	1 (1.0)	1 (100)	0 (-)		1 (100)	0 (-)	
TNM stages	99			.932			.055
I	22 (22.4)	15 (68.2)	7 (31.8)		15 (68.2)	7 (31.8)	
II	36 (36.7)	24 (66.7)	12 (33.3)		18 (50.0)	18 (50.0)	
III	39 (39.8)	24 (61.5)	15 (38.5)		14 (35.9)	25 (64.1)	
IV	1 (1.0)	1 (100)	0 (-)		1 (100)	0 (-)	
Neoadjuvant therapy	99			.012			.878
Present	15 (15.2)	14 (93.3)	1 (6.7)		7 (46.7)	8 (53.3)	
Absent	84 (84.8)	50 (59.5)	34 (40.5)		41 (48.8)	43 (51.2)	
Death	99			<.001			.384
Present	25 (25.3)	24 (96.0)	1 (4.0)		14 (56.0)	11 (44.0)	
Absent	74 (74.7)	40 (54.1)	34 (45.9)		34 (45.9)	40 (54.1)	
Recurrence	99			.001			.872
Present	22 (22.2)	21 (95.5)	1 (4.5)		11 (50.0)	11 (50.0)	
Absent	77 (77.8)	43 (55.8)	34 (44.2)		37 (48.1)	40 (51.9)	
Histologic type	99			.135			.037
Invasive ductal carcinoma, NOS	77 (77.8)	52 (67.5)	25 (32.5)		35 (45.5)	42 (54.5)	
Invasive ductal carcinoma with basal-like phenotype	16 (16.2)	10 (62.5)	6 (37.5)		12 (75.0)	4 (25.0)	
Invasive ductal carcinoma with medullary pattern	5 (5.1)	1 (20.0)	4 (80.0)		1 (20)	4 (80)	
Invasive ductal carcinoma with metaplastic features	1 (1.0)	1 (100)	0 (-)		0 (-)	1 (100)	
Receptor expression	98			.116			.001
Triple (-)	51 (52.0)	37 (72.5)	14 (27.5)		34 (66.7)	17 (33.3)	
HR (-)/HER2 (+)	23 (23.5)	11 (47.8)	12 (52.2)		5 (21.7)	18 (78.3)	
HR (+)/HER2 (+)	24 (24.5)	16 (66.7)	8 (33.3)		9 (37.5)	15 (62.5)	
Histologic grade	99			.538			.742
1	2 (2.0)	2 (100)	0 (-)		1 (50.0)	1 (50.0)	
2	9 (9.1)	5 (55.6)	4 (44.4)		3 (33.3)	6 (66.7)	
3	88 (88.9)	57 (64.8)	31 (35.2)		44 (50.0)	44 (50.0)	
ER expression	99			.748			.172
Negative (0)	72 (72.7)	48 (66.7)	24 (33.3)		39 (54.2)	33 (45.8)	
Low (1-10%)	5 (5.1)	3 (60.0)	2 (40.0)		2 (40.0)	3 (60.0)	
High (>10%)	22 (22.2)	13 (59.1)	9 (40.9)		7 (31.8)	15 (68.2)	

(*continued on next page*)

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Table 3 (*continued*)

		PD-1 Expression on iTIL, n (%)			PD-1 Expression on sTIL, n (%)		
	n (%)	Low (< 5%)	High (≥ 5%)	*P*	Low (<5%)	High (≥5%)	*P*
PR expression	98			.811			.249
Negative (0)	69 (70.4)	45 (65.2)	24 (34.8)		34 (49.3)	35 (50.7)	
Low (1%-20%)	15(15.3)	9 (60.0)	6 (40.0)		5 (33.3)	10 (66.7)	
High (> 20%)	14 (14.3)	10 (71.4)	4 (28.6)		9 (64.3)	5 (35.7)	
HER2 overexpression/ amplification (IHC and ISH)	98			.117			<.001
Present	47 (48)	27 (57.4)	20 (42.6)		14 (29.8)	33 (70.2)	
Absent	51 (52)	37 (72.5)	14 (27.5)		34 (66.7)	17 (33.3)	
Ki67 proliferative index	39			.514			1.000
Low (1%-15%)	2 (5.1)	2 (100)	0 (-)		1 (50.0)	1 (50.0)	
High (> 15%)	37 (94.9)	22 (59.5)	15 (40.5)		22 (59.5)	15 (40.5)	
Density of sTIL	99			<.001			.002
Mild (1%-10%)	44 (44.4)	38 (86.4)	6 (13.6)		29 (65.9)	15 (34.1)	
Moderate (11%-49%)	29 (29.3)	20 (69.0)	9 (31.0)		13 (44.8)	16 (55.2)	
High (≥ 50%)	26 (26.3)	6 (23.1)	20 (76.9)		6 (23.1)	20 (76.9)	
TLS	99			.001			.108
Present	14 (14.1)	3 (21.4)	11 (78.6)		4 (28.6)	10 (71.4)	
Absent	85 (85.9)	61 (71.8)	24 (28.2)		44 (51.8)	41 (48.2)	
Lymphoid aggregate	99			.099			.283
Present	30 (30.3)	23 (76.7)	7 (23.3)		17 (56.7)	13 (43.3)	
Absent	69 (69.7)	41 (59.4)	28 (40.6)		31 (44.9)	38 (55.1)	
Intensity of inflammation in non-tumoral breast parenchyma	75			.784			.114
Mild (1%-10%)	40 (53.3)	23 (57.5)	17 (42.5)		13 (32.5)	27 (67.5)	
Moderate (11%-49%)	21 (28.0)	11 (52.4)	10 (47.6)		9 (42.9)	12 (57.1)	
High (≥ 50%)	14 (18.7)	9 (64.3)	5 (35.7)		9 (64.3)	5 (35.7)	
Accompanying DCIS	99			.123			.316
Present	38 (38.4)	21 (55.3)	17 (44.7)		16 (42.1)	22 (57.9)	
Absent	61 (61.6)	43 (70.5)	18 (29.5)		32 (52.5)	29 (47.5)	
TIL density in accompanying DCIS	38			.105			.573
Mild (1%-10%)	16 (42.1)	11 (68.8)	5 (31.3)		8 (50.0)	8 (50.0)	
Moderate (11%-49%)	9 (23.7)	6 (66.7)	3 (33.3)		4 (44.4)	5 (55.6)	
High (≥ 50%)	13 (34.2)	4 (30.8)	9 (69.2)		4 (30.8)	9 (69.2)	

Itil = Intratumoral tumor infiltrating lymphocytes; sTIL = Stromal tumor infiltrating lymphocytes; IHC = Immunohistochemistry; ISH = In situ hybridization

TIM-3 expression was observed on stromal TIL in those cases with moderate to high stromal TIL density and with TLS (P= .050 and P= .009, respectively).

In univariate analyzes performed with the Kaplan-Meier method, a statistically significant relationship was found between PD-1 expression in the intratumoral TIL and overall survival and disease-free survival (P< .001 and P= .001, respectively) (Figure 1). Overall survival and disease-free survival also tend to be higher in cases with high PD-1 expression on intratumoral TIL. However, no significant relationship was found between survival and other marker expressions on TIL (ie, PD-1 expression on stromal TIL, LAG-3 and TIM-3 expression on both intratumoral and stromal TIL). Nevertheless, patients with higher expression of these receptors tend to have higher overall survival and disease-free survival rates. These results were evaluated with the univariate Cox regression test. Among these 3 biomarkers, a statistically significant difference was found only in relation to PD-1. This difference appeared as higher PD-1 expression on intratumoral TIL associates with higher overall (HazR = 0.07, 95% CI 0.009-0.504, P= .009) and also disease-free survival rates (HazR = 0.07, 95% CI 0.01-0.5, P= .011). In the multivariate Cox regression test which was performed to investigate PD-1 expression on intratumoral TIL in terms of independent positive prognostic factor, a statistically significant difference was found between PD-1 expression on intratumoral TIL in terms of overall survival (HazR = 0.05, 95% CI 0.006-0.4, P= .005), as well as disease-free survival (HazR = 0.06, 95% CI 0.007- 0.5, P= .010).

Discussion

In terms of high-grade breast carcinomas which this study was conducted, HR (-)/HER2 (+) and triple (-) invasive ductal carcinomas, as well as those showing basal-like phenotype, were shown

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Table 4 The Relationship Between LAG-3 Expression on iTIL and sTIL With Clinicopathological and Histological Parameters in Invasive Breast Tumors

Parameters	Patients	LAG-3 Expression on iTIL, n (%)			LAG-3 Expression on sTIL, n (%)		
	n (%)	Low (<5%)	High (≥ 5%)	*P*	Low (<5%)	High (≥ 5%)	*P*
Age at diagnosis	99			.129			.383
< 40	16 (16.2)	9 (56.3)	7 (43.8)		6 (37.5)	10 (62.5)	
≥ 40	83 (83.8)	63 (75.9)	20 (24.1)		41 (49.4)	42 (50.6)	
pT	97			.115			.140
pT1	28 (28.9)	21 (75.0)	7 (25.0)		16 (57.1)	12 (42.9)	
pT2	58 (59.8)	44 (75.9)	14 (24.1)		27 (46.6)	31 (53.4)	
pT3	10 (10.3)	4 (40.0)	6 (60.0)		2 (20.0)	8 (80.0)	
pT4	1 (1.0)	1 (100)	0 (-)		0 (-)	1 (100)	
pN	98			.442			.145
pN0	39 (39.8)	31 (79.5)	8 (20.5)		21 (53.8)	18 (46.2)	
pN1	23 (23.5)	14 (60.9)	9 (39.1)		6 (26.1)	17 (73.9)	
pN2	16 (16.3)	11 (68.8)	5 (31.3)		9 (56.3)	7 (43.8)	
pN3	20 (20.4)	15 (75.0)	5 (25.0)		10 (50.0)	10 (50.0)	
pM	98			1.000			1.000
pM0	97 (99.0)	70 (72.2)	27 (27.8)		46 (47.4)	51 (52.6)	
pM1	1 (1.0)	1 (100)	0 (-)		0 (-)	1 (100)	
TNM stages	99			.834			.679
I	22 (22.4)	17 (77.3)	5 (22.7)		12 (54.5)	10 (45.5)	
II	36 (36.7)	26 (72.2)	10 (27.8)		15 (41.7)	21 (58.3)	
III	39 (39.8)	27 (69.2)	12 (30.8)		19 (48.7)	20 (51.3)	
IV	1 (1.0)	1 (100)	0 (-)		0 (-)	1 (100)	
Neoadjuvant therapy	99			.062			.622
Present	15 (15.2)	14 (93.3)	1 (6.7)		8 (53.3)	7 (46.7)	
Absent	84 (84.8)	58 (69.0)	26 (31.0)		39 (46.4)	45 (53.6)	
Death	99			.143			.323
Present	25 (25.3)	21 (84.0)	4 (16.0)		14 (56.0)	11 (44.0)	
Absent	74 (74.7)	51 (68.9)	23 (31.1)		33 (44.6)	41 (55.4)	
Recurrence	99			.278			0.216
Present	22 (22.2)	18 (81.8)	4 (18.2)		13 (59.1)	9 (40.9)	
Absent	77 (77.8)	54 (70.1)	23 (29.9)		34 (44.2)	43 (55.8)	
Histologic type	99			.001			.015
Invasive ductal carcinoma, NOS	77 (77.8)	61 (79.2)	16 (20.8)		42 (54.5)	35 (45.5)	
Invasive ductal carcinoma with basal-like phenotype	16 (16.2)	11 (68.8)	5 (31.3)		5 (31.3)	11 (68.8)	
Invasive ductal carcinoma with medullary pattern	5 (5.1)	0 (-)	5 (100)		0 (-)	5 (100)	
Invasive ductal carcinoma with metaplastic features	1 (1.0)	0 (-)	1 (100)		0 (-)	1 (100)	
Receptor expression	98			.740			.764
Triple (-)	51 (52.0)	37 (72.5)	14 (27.5)		23 (45.1)	28 (54.9)	
HR (-)/HER2 (+)	23 (23.5)	16 (69.6)	7 (30.4)		11 (47.8)	12 (52.2)	
HR (+)/HER2 (+)	24 (24.5)	19 (79.2)	5 (20.8)		13 (54.2)	11 (45.8)	
Histologic grade	99			.478			.378
1	2 (2.0)	2 (100)	0 (-)		2 (100)	0 (-)	
2	9 (9.1)	8 (88.9)	1 (11.1)		5 (55.6)	4 (44.4)	
3	88 (88.9)	62 (70.5)	26 (29.5)		40 (45.5)	48 (54.5)	
ER expression	99			.775			.419
Negative (0)	72 (72.7)	53 (73.6)	19 (26.4)		34 (47.2)	38 (52.8)	
Low (1%-10%)	5 (5.1)	3 (60.0)	2 (40.0)		1 (20.0)	4 (80.0)	
High (>10%)	22 (22.2)	16 (72.7)	6 (27.3)		12 (54.5)	10 (45.5)	

(*continued on next page*)

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Table 4 (*continued*)

Parameters	Patients	LAG-3 Expression on iTIL, n (%)			LAG-3 Expression on sTIL, n (%)		
	n (%)	Low (<5%)	High (≥ 5%)	*P*	Low (<5%)	High (≥ 5%)	*P*
PR expression	98			.437			.378
Negative (0)	69 (70.4)	52 (75.4)	17 (24.6)		32 (46.4)	37 (53.6)	
Low (1%-20%)	15(15.3)	9 (60.0)	6 (40.0)		6 (40.0)	9 (60.0)	
High (> 20%)	14 (14.3)	11 (78.6)	3 (21.4)		9 (64.3)	5 (35.7)	
HER2 overexpression/ amplification (IHC and ISH)	98			.830			.555
Present	47 (48.0)	35 (74.5)	12 (25.5)		24 (51.1)	23 (48.9)	
Absent	51 (52.0)	37 (72.5)	14 (27.5)		23 (45.1)	28 (54.9)	
Ki67 proliferative index	39			1.000			1.000
Low (1%-15%)	2 (5.1)	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
High (> 15%)	37 (94.9)	24 (64.9)	13 (35.1)		13 (35.1)	24 (64.9)	
Density of sTIL	99			<.001			<.001
Mild (1%-10%)	44 (44.4)	42 (95.5)	2 (4.5)		31 (70.5)	13 (29.5)	
Moderate (11%-49%)	29 (29.3)	23 (79.3)	6 (20.7)		13 (44.8)	16 (55.2)	
High (≥ 50%)	26 (26.3)	7 (26.9)	19 (73.1)		3 (11.5)	23 (88.5)	
TLS	99			.002			.035
Present	14 (14.1)	5 (35.7)	9 (64.3)		3 (21.4)	11 (78.6)	
Absent	85 (85.9)	67 (78.8)	18 (21.2)		44 (51.8)	41 (48.2)	
Lymphoid aggregate	99			.284			.586
Present	30 (30.3)	24 (80.0)	6 (20.0)		13 (43.3)	17 (56.7)	
Absent	69 (69.7)	48 (69.6)	21 (30.4)		34 (49.3)	35 850.7)	
Intensity of inflammation in non-tumoral breast parenchyma	75			.671			.577
Mild (1%-10%)	40 (53.3)	30 (75.0)	10 (25.0)		22 (55.0)	18 (45.0)	
Moderate (11%-49%)	21 (28.0)	14 (66.7)	7 (33.3)		9 (42.9)	12 (57.1)	
High (≥ 50%)	14 (18.7)	9 (64.3)	5 (35.7)		6 (42.9)	8 (57.1)	
Accompanying DCIS	99			.448			.667
Present	38 (38.4)	26 (68.4)	12 (31.6)		17 (44.7)	21 (55.3)	
Absent	61 (61.6)	46 (75.4)	15 (24.6)		30 (49.2)	31 (50.8)	
TIL density in accompanying DCIS	38			.072			.117
Mild (1%-10%)	16 (42.1)	14 (87.5)	2 (12.5)		10 (62.5)	6 (37.5)	
Moderate (11%-49%)	9 (23.7)	6 (66.7)	3 (33.3)		4 (44.4)	5 (55.6)	
High (≥ 50%)	13 (34.2)	6 (46.2)	7 (53.8)		3 (23.1)	10 (76.9)	

Itil = Intratumoral tumor infiltrating lymphocytes; sTIL = Stromal tumor infiltrating lymphocytes; IHC = Immunohistochemistry; ISH = In situ hybridization

to have higher stromal TIL density. In comparison, HR (+) breast carcinomas were strikingly found to have lower TIL density, regardless of HER2 status. Similarly, studies in the literature report that invasive ductal carcinomas with basal-like phenotype, triple (-), and HER2 (+) tumors have higher TIL density.[17-20] It has also been reported that the basal-like phenotype that occurs in breast carcinomas is associated with increased mutation burden. Likewise, triple (-) and HER2 (+) groups were found to have a higher tumor mutation burden compared to luminal groups.[21] It is known that more inflammatory cell infiltration occurs in the tumor microenvironment due to increased neoantigens in tumors with increased mutation burden.[6] It has also been shown that there is more pro-inflammatory cytokine [tumor necrosis factor-alpha (TNF-α) and interleukin-beta (IL-β)] release in basal-like phenotype breast cancers than in luminal A cancers.[22] These data explain the high TIL density in cases diagnosed with basal-like invasive ductal carcinoma and in HER2 (+) cases.

In the classification made considering only HER2, no significant relationship was found between stromal TIL density and HER2 expression. In some studies, it is reported that the molecular intrinsic subtype with the highest mutation burden is the HER2 dominant subtype.[21] However, this molecular subtype is a heterogeneous group in terms of immunohistochemical expressions of ER, PR, and HER2, and it is difficult to determine this molecular subtype by taking into account only the HER2 expression.[2,23] To give an example, in a gene expression analysis study including PAM50, Prat et al. found that %44 of HER2 (+) tumors were immunohistochemically compatible with HER2 dominant, %26.8 luminal B, 17.6% luminal A, and 11% basal-like subtypes.[2] This heterogeneity may be the reason why no significant relationship

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Table 5 The Relationship Between TIM-3 Expression on iTIL and sTIL With Clinicopathological and Histological Parameters in Invasive Breast Tumors

Parameters	Patients	TIM-3 Expression on iTIL n (%)			TIM-3 Expression on sTIL n (%)		
	n (%)	Low (< 5%)	High (≥ 5%)	*P*	Low (< 5%)	High (≥ 5%)	*P*
Age at diagnosis	99			.234			1.000
< 40	16 (16.2)	12 (75.0)	4 (25.0)		15 (93.8)	1 (6.3)	
≥ 40	83 (83.8)	73 (88.0)	10 (12.0)		76 (91.6)	7 (8.4)	
pT	97			.730			1.000
pT1	28 (28.9)	25 (89.3)	3 (10.7)		26 (92.9)	2 (7.1)	
pT2	58 (59.8)	49 (84.3)	9 (15.5)		53 (91.4)	5 (8.6)	
pT3	10 (10.3)	8 (80.0)	2 (20.0)		9 (90.0)	1 (10.0)	
pT4	1 (1.0)	1 (100)	0 (-)		1 (100)	0 (-)	
pN	98			.580			.439
pN0	39 (39.8)	32 (82.1)	7 (17.9)		35 (89.7)	4 (10.3)	
pN1	23 (23.5)	19 (82.6)	4 (17.4)		20 (87.0)	3 (13.0)	
pN2	16 (16.3)	14 (87.5)	2 (12.5)		15 (93.8)	1 (6.3)	
pN3	20 (20.4)	19 (95.0)	1 (5.0)		20 (100)	0 (-)	
pM	98			1.000			1.000
pM0	97 (99.0)	83 (85.6)	14 (14.4)		89 (91.8)	8 (8.2)	
pM1	1 (1.0)	1 (100)	0 (-)		1 (100)	0 (-)	
TNM stages	99			.597			.665
I	22 (22.4)	19 (86.4)	3 (13.6)		20 (90.9)	2 (9.1)	
II	36 (36.7)	29 (80.6)	7 (19.4)		32 (88.9)	4 (11.1)	
III	39 (39.8)	35 (89.7)	4 (10.3)		37 (94.9)	2 (5.1)	
IV	1 (1.0)	1 (100)	0 (-)		1 (100)	0 (-)	
Neoadjuvant therapy	99			.119			.603
Present	15 (15.2)	15 (100)	0 (-)		15 (100)	0 (-)	
Absent	84 (84.8)	70 (83.3)	14 (16.7)		76 (90.5)	8 (9.5)	
Death	99			.110			.675
Present	25 (25.3)	24 (96.0)	1 (4.0)		24 (96.0)	1 (4.0)	
Absent	74 (74.7)	61(82.4)	13 (17.6)		67 (90.5)	7 (9.5)	
Recurrence	99			.183			.680
Present	22 (22.2)	21(95.5)	1 (4.5)		21 (95.5)	1 (4.5)	
Absent	77 (77.8)	64 (83.1)	13 (16.9)		70 (90.9)	7 (9.1)	
Histologic type	99			.001			.007
Invasive ductal carcinoma, NOS	77 (77.8)	71 (92.2)	6 (7.8)		74 (96.1)	3 (3.9)	
Invasive ductal carcinoma with basal-like phenotype	16 (16.2)	12 (75.0)	4 (25.0)		13 (81.3)	3 (18.8)	
Invasive ductal carcinoma with medullary pattern	5 (5.1)	2 (40.0)	3 (60.0)		4 (80.0)	1 (20.0)	
Invasive ductal carcinoma with metaplastic features	1 (1.0)	0 (-)	1 (100)		0 (-)	1 (100)	
Receptor expression	98			.065			.89
Triple (-)	51 (52.0)	42 (82.4)	9 (17.6)		44 (86.5)	7 (13.7)	
HR (-)/HER2 (+)	23 (23.5)	19 (82.6)	4 (17.4)		22 (95.7)	1 (4.3)	
HR (+)/HER2 (+)	24 (24.5)	24 (100)	0 (-)		24 (100)	0 (-)	
Histologic grade	99			.527			1.000
1	2 (2.0)	2 (100)	0 (-)		2 (100)	0 (-)	
2	9 (9.1)	9 (100)	0 (-)		9 (100)	0 (-)	
3	88 (88.9)	74 (84.1)	14 (15.9)		80 (90.9)	8 (9.1)	
ER expression	99			.565			.281
Negative (0)	72 (72.7)	61 (84.7)	11 (15.3)		64 (88.9)	8 (11.1)	
Low (1%-10%)	5 (5.1)	4 (80.0)	1 (20.0)		5 (100)	0 (-)	
High (> 10%)	22 (22.2)	20 (90.9)	2 (9.1)		22 (100)	0 (-)	

(*continued on next page*)

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Table 5 (*continued*)

Parameters	Patients	TIM-3 Expression on iTIL n (%)			TIM-3 Expression on sTIL n (%)		
	n (%)	Low (< 5%)	High (≥ 5%)	*P*	Low (< 5%)	High (≥ 5%)	*P*
PR expression	98			.397			.297
Negative (0)	69 (70.4)	58 (84.1)	11 (15.9)		61 (88.4)	8 (11.6)	
Low (1%-20%)	15(15.3)	13 (86.7)	2 (13.3)		15 (100)	0 (-)	
High (> 20%)	14 (14.3)	14 (100)	0 (-)		14 (100)	0 (-)	
HER2 overexpression/ amplification (IHC and ISH)	98			.183			.061
Present	47 (48.0)	43 (91.5)	4 (8.5)		46 (97.9)	1 (2.1)	
Absent	51 (52.0)	42 (82.4)	9 (17.6)		44 (86.3)	7 (13.7)	
Ki67 proliferative index	39			.413			.287
Low (1%-15%)	2 (5.1)	1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)	
High (> 15%)	37 (94.9)	29 (78.4)	8 (21.6)		32 (86.5)	5 (13.5)	
Density of stromal TIL	99			<.001			<.001
Mild (1%-10%)	44 (44.4)	44 (100)	0 (-)		44 (100)	0 (-)	
Moderate (11%-49%)	29 (29.3)	28 (96.6)	1 (3.4)		29 (100)	0 (-)	
High (≥ 50%)	26 (26.3)	13 (50.0)	13 (50.0)		18 (69.2)	8 (30.8)	
TLS	99			<.001			.013
Present	14 (14.1)	7 (50.0)	7 (50.0)		10 (71.4)	4 (28.6)	
Absent	85 (85.9)	78 (91.8)	7 (8.2)		81 (95.3)	4 (4.7)	
Lymphoid aggregate	99			.058			.429
Present	30 (30.3)	29 (96.7)	1 (3.3)		29 (96.7)	1 (3.3)	
Absent	69 (69.7)	56 (81.2)	13 (18.8)		62 (89.9)	7 (10.1)	
Intensity of inflammation in non-tumoral breast parenchyma	75			.355			.038
Mild (1%-10%)	40 (53.3)	35 (87.5)	5 (12.5)		38 (95.0)	2 (5.0)	
Moderate (11%-49%)	21 (28.0)	17 (81.0)	4 (19.0)		20 (95.2)	1 (4.8)	
High (≥ 50%)	14 (18.7)	10 (71.4)	4 (28.6)		10 (71.4)	4 (28.6)	
Accompanying DCIS	99			.825			1.000
Present	38 (38.4)	33 (86.8)	5 (13.2)		35 (92.1)	3 (7.9)	
Absent	61 (61.6)	52 (85.2)	9 (14.8)		56 (91.8)	5 (8.2)	
TIL density in accompanying DCIS	38			.415			.310
Mild (1%-10%)	16 (42.1)	15 (93.8)	1 (6.3)		16 (100)	0 (-)	
Moderate (11%-49%)	9 (23.7)	8 (88.9)	1 (11.1)		8 (88.9)	1 (11.1)	
High (≥ 50%)	13 (34.2)	10 (76.9)	3 (23.1)		11 (84.6)	2 (15.4)	

Itil = Intratumoral tumor infiltrating lymphocytes; sTIL = Stromal tumor infiltrating lymphocytes; IHC = Immunohistochemistry; ISH = In situ hybridization

could be shown in our study in terms of TIL density when only classification by HER2 expression considered.

There are many studies in the literature that have investigated the relationship between TIL and survival in breast cancer population with different clinical characteristics.[17,18,24-27] Of these studies, Denkert et al. had the largest number of cases with 3771 breast cancer cases that received neoadjuvant therapy in 2018. In this study, which evaluated core biopsies before neoadjuvant therapy, it was found that overall survival also increased with an increase in TIL density in triple (-) breast cancers, while no significant association was found in HER2 (+) breast tumors. However, overall survival has been reported to decrease with an increase in TIL in luminal HER2 (-) breast tumors. It was also mentioned that disease-free survival in triple (-) breast tumors and HER2 (+) breast tumors increased with the increase of TIL, and no significant relationship was found in this regard in luminal HER2 (-) breast tumors[17] In our study, although no significant difference was found in terms of overall survival and disease-free survival with the groups determined as mild-moderate-high stromal TIL regarding all cases, it was found that the group with mild TIL had lower overall and disease-free survival. In the analyzes performed according to the receptor expressions, unlike the above-mentioned study of Denkert et al. a significant relationship was found between the overall survival in the HR (-)/HER2 (+) group and the stromal TIL density rates, and it was determined that those patients with high TIL density had longer overall survival. In multivariate survival analyzes performed by combining moderate and high TIL density groups (as moderate/high vs. mild), stromal TIL density was found to be an independent prognostic factor in terms of overall survival and disease-free survival. This result was interpreted as "high stromal TIL density in high-grade breast carcinomas is an indicator of good prognosis."

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In addition to TIL, another subject that has been investigated in recent years, is the relation of TLS, which are ectopic lymphoid formations that may accompany the tumor and resemble the secondary lymphoid organs, with prognosis.[10] In many studies, the presence of TLS formation in different cancers (lung cancer, colon cancer, and melanoma) has been investigated in terms of prognosis.[11-13] In our study, it was observed that the presence of TLS in breast tumors was more common in those with high TIL density. This finding is also mentioned in several studies in the literature.[14,28-30]

When we look at the receptor expression status, the TLS were most frequently observed in the HR (-)/HER2 (+) group and followed by in the triple (-) group. We also found a significant association between the presence of TLS and overall survival in univariate analyses determining that overall survival was higher in the presence of TLS. Regarding TLS and survival, different outcomes were mentioned in literature. Such that, in 2 studies, when all molecular intrinsic subtypes of breast carcinomas were evaluated together, no relationship was found between TLS and survival[14,29]; but, when Liu et al. evaluated HER2 (+) breast tumors separately, they found that TLS was associated with longer disease-free survival.[14]

PD-1, LAG-3, and TIM-3, three less investigated immune checkpoint molecules which our study focuses on, are targets that may be promising in immunotherapy. In our study, we examined the relationship of these receptors with prognostic parameters and also with survival in breast cancer patients with high tumor grade. We concluded that all these immune checkpoint inhibitor receptors are highly expressed in tumors with high stromal TIL density and in tumors containing TLS.

Considering PD-1 expression, higher PD-1 expression was found in HER2 (+) tumors, regardless of HR status. This result was found different from some studies in the literature which have reported that stromal and intratumoral high PD-1 expression is more common in triple (-) and basal-like invasive breast tumors.[31-34] We also found that high PD-1 expression in intratumoral TIL was associated with increased overall and disease-free survival. In multivariate analyzes, it was determined that PD-1 expression in intratumoral TIL was an independent positive prognostic factor in terms of overall and disease-free survival. It was also noted that some studies suggested that there was no significant relationship between PD-1 expression and overall survival and disease-free survival.[31,32,35,36] On the other hand, Yeong et al. reported that increased PD-1 expression was associated with increased overall survival and disease-free survival, in their study including 269 triple (-) breast carcinoma cases.[37] In contrast to this study and to ours, there are also several studies in the literature reporting that PD-1 expression is associated with decreased overall and disease-free survival.[34,38]

In our study, high LAG-3 expression on intratumoral and stromal TIL was tended to be higher in patients in terms of survival, but no statistically significant relationship could be shown.

There are only a few studies with different results in the literature investigating the relationship between survival and LAG-3 expression on TIL in breast cancer. In a study conducted by Bottai et al. concerning 259 triple (-) breast tumors, no statistically significant relationship was found between LAG-3 and survival.[36] Tu et al. found that LAG-3 expression improves post-progression survival in triple (-) breast tumors and disease-free survival in those with a p53 mutation.[39] In their study, Wang et al. were reported that high LAG-3 expression after neoadjuvant treatment in triple (-) breast tumors is associated with poor prognosis.[40] In an animal model with mice evaluating treatment results, co-inhibition of PD-1 and LAG-3 was found to be correlated with a better response to the tumor in triple (-) breast tumors.[41] On the other hand, in a clinical study, Brignone et al. were reported that combined use of paclitaxel and IMP321 (LAG-3 antagonist) in metastatic breast cancer has provided an objective tumor response of 50%.[3] LAG-3 is thought to be a strong immunotherapy target, although the relationship between LAG-3 expression and survival on TIL has not yet been definitively explained.

Similar to LAG-3, high TIM-3 expression on intratumoral and stromal TIL was tended to be higher in patients in terms of survival, but no statistically significant correlation could be shown. When looking at the small number of similar studies available in the literature, it was seen that similar results were reported. In the study of Burugu et al. including 3992 breast cancer cases, TIM-3 expression on intratumoral TIL was found to be an independent positive prognostic factor.[42] It has also been reported that high TIM-3 expression in triple (-) breast tumors is associated with increased disease-free survival.[39,43] Only 1 study reported that TIM-3 expression in tumor cells may cause tumor progression.[44] No clinical studies have yet been found in the literature investigating the therapeutic efficacy of TIM-3 antagonists in breast tumors. Similar to LAG-3 and PD-1, TIM-3 may be one of the targets to be used for immunotherapy in the near future.

Regarding DCIS, which is another important tumor type usually forming a breast lump with indistinct borders, studies by evaluating TIL were also conducted in recent years in order to get some information about the prognosis of those patients.[45-48] In our study, high stromal TIL density rate was found in 8.8% of DCIS, especially in grade 3 tumors; however, such TIL density was not seen in those with microinvasion. Besides, no statistically significant relationship was found between TIL density and microinvasion. In the study of Beguinot et al. in which microinvasive DCIS and pure DCIS were compared, it was found that microinvasive carcinomas have higher TIL density; however, it has been reported that the difference is not statistically significant.[48-49]

The healing phenomenon was first described in 1934 by Muir et al. as the spontaneous disappearance of DCIS and its replacement by fibrous tissue.[50] This condition is characterized by inflammation and fibrosis of the periductal stroma containing the tumor.[51] In our study, a significant relationship was found between the healing phenomenon and TIL density in DCIS. Forty percent of cases with sign of healing phenomenon were included in high TIL density group, while only 2.4% of cases not showing this feature were in this group. Similar results were also noted by Morita et al.[52]

In our study, 2 of 68 patients with DCIS died of non-tumor causes and no recurrence was observed during the follow-up period. Therefore, survival analysis could not be performed in the DCIS group. However, in a study including 1488 DCIS cases which was conducted by Pruneri et al., it was showed that 245 patients recurred as in situ or as invasive tumors during follow-up, and there was no significant relationship between stromal TIL and recurrence[45].

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In our study, PD-1 expression on intratumoral TIL was not associated with any of the clinical and histopathological parameters in DCIS. However, PD-1 expression on stromal TIL was found to be related to TIL density determining that tumors with high stromal TIL density show a higher rate of PD-1 expression. Recently, Ubago et al. reported that 31% of HER2 (+) DCIS have PD-1 expression on TIL.[53] Regarding LAG-3 and TIM-3 expression in intratumoral and stromal TIL, no significant association was found with any of the clinical or histopathological parameters in DCIS. No study has been found on this new subject in the literature yet.

Conclusion

As a conclusion, the relationship between TIL, as well as TLS and prognostic factors is an important and significant issue particularly in high grade invasive tumors. Stating the percentage of TIL and the presence of TLS in pathology reports will help to predict the prognosis of the patient and also to contribute the treatment modality. Another important issue we would like to mention is that PD-1 expression on intratumoral TIL is a positive prognostic factor in terms of survival in invasive breast cancer. Other receptor expressions, such as LAG-3 and TIM-3, also may indicate good prognosis; however, further studies on these new markers are needed. Immunotherapy seems to be a promising treatment option for those patients with high grade tumors and whose tumors show marked immune response and express these novel immune checkpoint molecules on TIL.

Clinical Practice Point

- Stromal TIL density was found to be an independent prognostic factor in terms of overall survival and disease-free survival in high-grade breast carcinomas.
- PD-1 expression on intratumoral TIL in invasive breast carcinomas was an independent positive prognostic factor in terms of overall and disease-free survival.
- High LAG-3 and TIM-3 expression on intratumoral and stromal TIL was tended to be higher in patients in terms of survival.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval

The study was approved by the Gazi University Faculty of Medicine Clinical Research Ethics Committee in terms of ethics on April 29, 2019, with the meeting number 04.

Author Contributions

E.A., G.E. and, A.D. performed study concept and design, development of methodology; E.A, G.E and, O.Y. performed development of writing and review of the paper; E.A. and G.E. provided acquisition, analysis and, interpretation of data, and statistical analysis. All authors read and approved the final paper.

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Disclosure

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clbc.2022.08.005.

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