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# CO-evaluation of Immunhistochemical PD-L1 and FOXP3 Expressions in Breast Cancer

## *Meme Tümörlerinde FOXP3 ve PDL1 Ekspresyonlarının Birlikte Değerlendirilmesi*

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## ABSTRACT

**Objective:** Breast cancer is the most common cancer among women and is the second most frequent cause of cancer-related deaths worldwide. The aim of this study was to determine the prognostic values of PD-L1 expression in breast cancers and to detect the presence of FOXP3-positive Treg cells in the tumor microenvironment.

**Methods:** This study included 210 females with breast cancer who had been histopathologically diagnosed in our hospital between 2011 and 2014.

**Results:** In this series, the mean age of the patients was 55.46 (12.5) years and they were followed up for a mean period of 61.9 (20.6) months. In only 6 cases (2.9%), there were weak membranous expressions of PD-L1 in tumor cells. However, PD-L1-positive inflammatory cells were seen in 15 tumors (7.1%). There was no significant relationship between PD-L1 expression and survival (p>0.05). In 14 (6.7%) cases, there were FOXP3-positive lymphocytes. The range of FOXP3-positive cells was between 1 and 30/HPF. There was no statistically significant association between survival times and the presence of Tregs (p>0.05).

**Conclusion:** In this study, no relation was found between PD-L1 positivity and molecular subtypes, histological grade, stage, and hormone receptor status of the breast tumor. There was no statistically significant relationship between FOXP3 and PD-L1 molecule and overall survival. We found a minimal positive effect of the presence of Treg inflammatory cells on survival. However, this relationship could not be proved by statistical analyzes

**Keywords:** PD-L1, FOXP3, Treg, breast cancers

## ÖZ

**Amaç:** Meme kanseri dünya genelinde kadınlar arasında en sık görülen kanserdir ve kansere bağlı ölümlerin ikinci sık nedenidir. Meme kanserlerinin tümörgenezinde PD-L1 ve FOXP3'ün rolleri göreceli olarak daha az değerlendirilen konulardır. Bu çalışmanın amacı, meme kanserlerinde PD-L1 ekspresyonlarının prognostik değerlerini belirlemek ve tümör mikro-çevresinde FOXP3 pozitif Treg hücrelerinin varlığını değerlendirmektir.

**Yöntem:** Bu çalışmaya, 2011-2014 yılları arasında hastanemizde tanı konulan 210 meme kanseri hastası dahil edildi.

**Bulgular:** Bu seride ortalama yaş 55,46 (12,5) yıl olup, hastalar ortalama 61,9 (20,6) ay boyunca takip edildi. Sadece 6 olguda (%2,9), tümör hücrelerinde zayıf PDL1 membranöz ekspresyonu vardı. Buna karşılık 15 olguda (%7,1) PD-L1 pozitif yangısal hücreler izlendi. PD-L1 ekspresyonu ile sağkalım arasında anlamlı ilişki bulunmadı (p>0,05). On dört (%6,7) olguda FOXP3 pozitif lenfosit vardı. FOXP3-pozitif hücre aralığı 1 ila 30/BBA arasındaydı. Sağkalım süresi ile Treg hücre varlığı arasında istatistiksel olarak

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anlamlı bir ilişki yoktu (p>0,05). Sağkalım ile nodal metastaz, pT evresi ve enflamatuvar karsinom varlığı (p<0,01) gibi bazı prognostik faktörler arasında istatistiksel olarak anlamlı ilişki saptandı.

**Sonuç:** Bu çalışmada PD-L1 pozitifliği ile meme tümörünün histolojik derecesi, evresi ve hormon reseptör durumuyla istatistiksel anlamlı ilişki saptanmamıştır. Hem FOXP3 hem de PD-L1 molekülünün genel sağkalım ile istatistiksel anlamlı ilişkisi saptanamamıştır. Ancak Treg enflamatuvar hücrelerin varlığının sağkalım üzerine minimal pozitif bir etkisi olduğu gösterilmekle birlikte bu ilişki istatistiksel analizlerle kanıtlanamamıştır.

**Anahtar Kelimeler:** PD-L1, FOXP3, Treg, meme kanseri

## INTRODUCTION

Invasive breast carcinoma is the most common malignancy in women, with a worldwide incidence of 23% among female malignancies, and with an incidence of 27% in developed countries.[1] Breast carcinoma is a heterogeneous disease with various morphological, molecular features and clinical results.[2] When invasive breast carcinomas are classified according to their immune phenotypes and molecular profiles; they are basically divided into 4 groups as luminal A, luminal B, HER2-positive and triple-negative carcinomas. However, in recent years, especially the tumors defined as triple-negative have been subclassified as basal-like and normal-like, and these subtypes have their own specific treatment approaches.[3,4]

The PD-1 (programed cell death-1) molecule is a receptor that is expressed on the surface of T, B, and NK cells and forces them to apoptosis by activating these cells. The ligand of this receptor (PD-L1) is expressed by tumor cells, forcing T, B cells, macrophages, and dendritic cells to apoptosis. It is expressed in many tumors such as breast, lung, stomach, colorectal cancer, hepatocellular, and renal cell carcinoma and reflects the poor prognosis.[5,6]

FOXP3 is a transcription factor expressed in regulatory T lymphocytes and is essential for their development and function. Treg cells play a role in suppressing the immune system. FOXP3 expression is considered a prognostic factor in tumors infiltrated by Treg cells. Studies have shown that PD-L1 and FOXP3 are pathways that work together and synergistically in breast carcinomas and allow the tumor to escape from the immune system induced by their expressions.[5]

The aim of this study was to evaluate the relationship between different clinicopathological variables of breast carcinomas and the expression of FOXP3 and PD-L1.

## METHODS

Patients diagnosed with breast carcinoma between January 2011 and December 2014 at our hospital who underwent mastectomy and breast excisional biopsy were included in this study. Patients whose slides could not be reached or whose immunohistochemical (IHC) staining could not be performed were excluded from the study.

The study was approved by the University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital of Local Ethics Committee (protocol number: 38, date: 11.12.2017).

Hematoxylin&Eosin (H&E) stained sections in the archives of the cases were re-evaluated according to 2012 World Health Organization classification of breast tumors. In terms of determining molecular subtypes, IHC determined c-erbB2 expressions were re-evaluated according to the ASCO/CAP 2013 guideline updates, and the cases among those hospitalized in 2011 and 2012 with c-erbB2 ratios between 10 and 30% and could not be confirmed with FISH were shifted to the HER2-positive group. From the pathology records, patients' ages at the time of diagnosis, follow-up periods, survival status, localization, size, stage, all similar features of the tumors, and all their histopathological examination results were re-evaluated. Detailed histopathological and IHC features of the tumors were documented.

The area that best reflects the tumor tissue and is most suitable for IHC staining was marked first on slides and then on blocks. Afterwards, cylindric paraffin tissue samples with a diameter of 2 mm were taken from the donor blocks with mapping-addressing technique using a manual microarray device and transferred to multiple blocks. Tissue samples obtained from the colon, spleen, and endometrium were used for addressing in multiple blocks.

Prior to this, H&E-stained sections were taken from each prepared block, and the presence of tumor in the sampled areas was confirmed. Then, 4 micrometer-thick sections were taken on poly-l-lysine-coated slides and stained manually with PD-L1 and FOXP3 antibodies using streptavidin-biotin peroxidase staining method (Invitrogen, Camarillo, 85-9043, CA, USA). Primary antibodies of PD-L1 (Abcam; ab205921-pd-l1) anti-FOXP3 (236A/E7) (Abcam, ab20034) were applied at 1/100 and 1/300 dilutions, respectively.

Stained slides were evaluated under a light microscope using three pathologists (A.C., G.D., G.G.). Membranous staining in tumor cells and cytoplasmic/membranous staining in inflammatory cells both performed for the detection of PD-L1 expression in tumor and accompanying inflammatory cells were evaluated separately and indicated as positive PD-L1 expression. For evaluation of FOXP3 expression, T lymphocytes with positively stained nuclei among tumor-infiltrating lymphocytes were counted. The number of these lymphocytes in a high power field (HPF)

where they were most densely observed was recorded. For both stainings, tonsillar tissue was used as a positive control. For FOXP3, there were internal control in tumors seen in areas other than tonsillar tissue.

### Statistical Analysis

The Statistical Package for the Social Sciences 22.0 program was used in the analysis of variables. The conformity of the data to the normal distribution was determined by the Shapiro-Wilk test and homogeneity of variance was evaluated with Levene. One-Way ANOVA, Kruskal-Wallis H tests were used compared with independent quantitative data of more than two groups. The Pearson chi-square test was used to compare categorical variables with each other. Kaplan-Meier-Log rank (Mantel-Cox) analysis was used to analyze the effect of PDL1 and FOXP3 expression on survival and survival rates. Variables were analyzed at 95% confidence level and p values less than 0.05 were considered significant.

## RESULTS

A total of 210 patients whose IHC expressions of PD-L1 and FOXP3 could be evaluated among patients who underwent mastectomy or excisional breast biopsy for breast carcinoma were included in this study. The ages of the patients at the time of diagnosis ranged from 30 to 85 years, and the mean age was 55.46 (12.5) years.

The mean follow-up period was 61.9 (20.6) months. One hundred-seventy (81%) patients survived, and 40 (19%) died.

Tumor localization was reported in 154 cases. Accordingly, 69 (44.8%) were located in the right breast and 84 (54.6%) in the left breast. The mean tumor diameter was 2.96 (0.6-16) cm. According to pathological T staging, the cases were distributed as follows: pT1 (n=91; 43.1%), pT2 (n=95; 45%), pT3 (n=7; 3.3%) and pT4 (n=18; 8.5%). The tumor was multifocal in 12 (8.5%), and unifocal in other cases. Histopathologically, tumors were classified as grade 1 in 13 (6.3%), grade 2 in 99 (47.1%), and grade 3 in 98 (46.6%) cases. *In situ* component was in 146 (73.4%) tumors.

Of the existing *in situ* components, 11 (7.2%) were comedies, 69 (47.1%) were noncomedoes, and 66 (45.7%) were comedo + noncomedo mixed *in situ* carcinoma type. Axillary lymph node dissection was performed in 152 (72.3%) cases, and lymph node metastasis was detected in 70 (46%) of these cases. In 51 (72.8%) cases with lymph node metastasis, capsular invasion was in the metastatic lymph nodes (Table 1). In IHC studies performed on 210 patients included in the study, ER-positivity was detected in 176 (83.8%) and PR-positivity in 157 (74.8%) cases. IHC, c-erbB2 was found to be 1 + or negative in 143 cases (68%), and both groups were considered HER2-negative. In the combined IHC-FISH evaluation, 46 cases (21.9%) were accepted as Her2 positive. Ki67 proliferation index was studied in all cases, and the mean Ki67 index was found to be 21.98±20.6.

Based on molecular classification, a number of cases with luminal A (n=66; 31.4%), luminal B (n=80; 38.1%), Her2-positive (n=46; 21.9%), and triple-negative (n=18; 8.6%) were detected. IHC, the accepted positivity for PD-L1 is membranous expression in tumor cells (Figure 1a) and in only 6 cases, PD-L1 was weakly expressed. In 15 cases, cytoplasmic PD-L1 expression was found in inflammatory cells (Figure 1b). The number of lymphocytes showing nuclear FOXP3-positive staining was determined per HPF in the area where these lymphocytes were mostly concentrated (Figure 2a, 2b). In 14 cases (6.7%) with nuclear expression, the number of FOXP3-positive lymphocytes varied between 1 and 30 mean 1.7 (3.6) in one HPF (Table 2).

No statistically significant relationship was not detected between the age and survival of the patients and PD-L1 expression (p=0.097, p=0.715). Similarly, no correlation was not detected between PD-L1 expression and tumor location (p=0.526), histological type (p=0.895), and grade (p=0.746), presence or type of *in situ* component (p=0.450, and p=0.121, respectively), axillary lymph node metastasis (p=0.333), lymph node capsule invasion (p=0.079), tumor size (p=0.729), and pathological tumor stage (p=0.270). Although cases with tumoral PD-L1 positivity in Kaplan-Meier survival analysis were the group with the shortest mean life span, this finding was not statistically significant due to the very limited number of PD-L1 positive cases (p=0.660).

Any statistically significant relationship was not detected between FOXP3 expression and the patient's age at the time of diagnosis (p=0.935), tumor localization (p=0.968), histological type of the tumor (p=0.741), tumor grade (p=0.203), presence or types of *in situ* components (p=0.472, and p=0.299, respectively), axillary lymph node metastasis (p=0.440), capsular invasion (p=0.383), tumor size (p=0.673), and pathological tumor stage (p=0.402).

Although the survival time was found to be slightly longer in cases with FOXP3-positive Treg cells any statistically significant correlation was not detected (p=0.077). Besides any statistically significant relationship was not found between the IHC determined ER-positivity according to PD-L1 and FOXP3 staining status.

The factors affecting survival statistically significantly were the presence of lymph node metastasis (p=0.006),

pathological tumor stage (p<0.01), epidermal invasion (p=0.014), and presence of tumor thrombus in dermal lymphatics in the samples examined (p<0.01), or in other words, the presence of inflammatory carcinoma.

## DISCUSSION

Studies have proven that the microenvironment of the tumor and its molecular properties are directly related to survival and prognosis in many tumors. While forming its own microenvironment, one of the most important features that facilitates escape of the tumor cell from the immune response is PD-L1 expression. In a study of 650 cases performed by Muenst et al.,[6] PD-L1 positivity was reported in 152 (23.4%) primary breast tumor cases and this positivity was related to the patient's age, tumor size, stage, grade and Ki67 status. Ghebeh et al.,[7] revealed that PD-L1 positivity was related to poor prognosis and they reported a significant relationship between PD-L1 positivity and the size of the tumor diameter greater than 4 cm (p=0.042) and the increased histological grade.

Li et al.[5] found PD-L1 positivity in 231 of 501 invasive breast carcinoma cases and reported that this positivity is a poor prognostic factor. In their study, they applied IHC dye to complete tissue blocks in 501 cases. In our study, the multiblock method was used, which evaluates a small area and does not give a definite idea about the whole tumor. This fact may be the reason for not detecting a

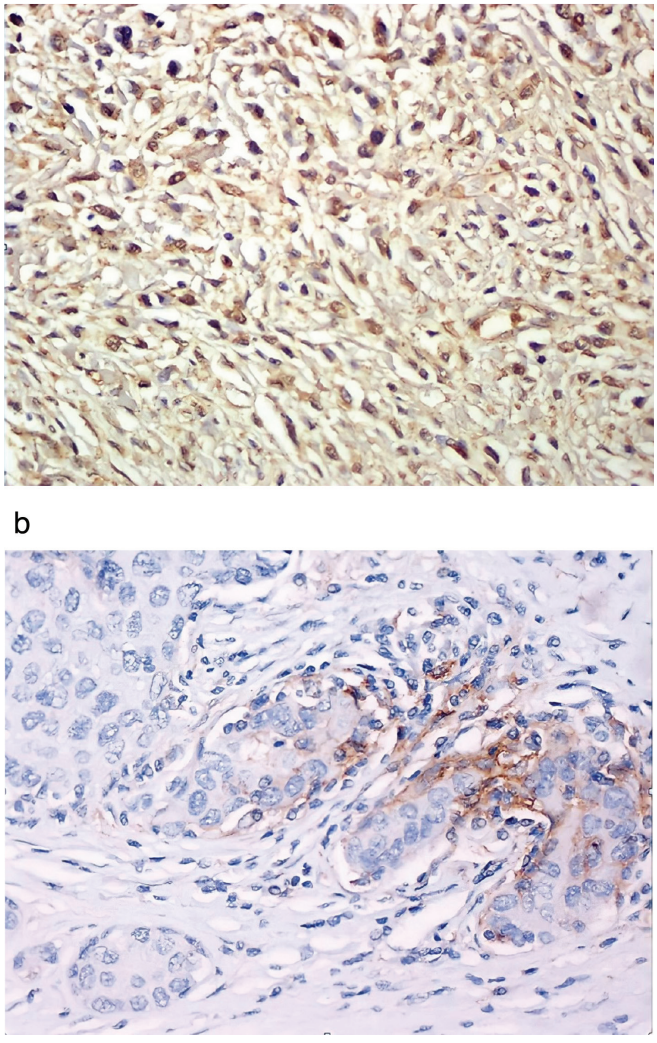


**Figure 1.** a) A case with immunhistochemically detected cytoplasmic PD-L1 positivity in inflammatory cells (DABx100). b) A case with immunhistochemically detected membranous PD-L1 positivity in tumor cells (DABx100)

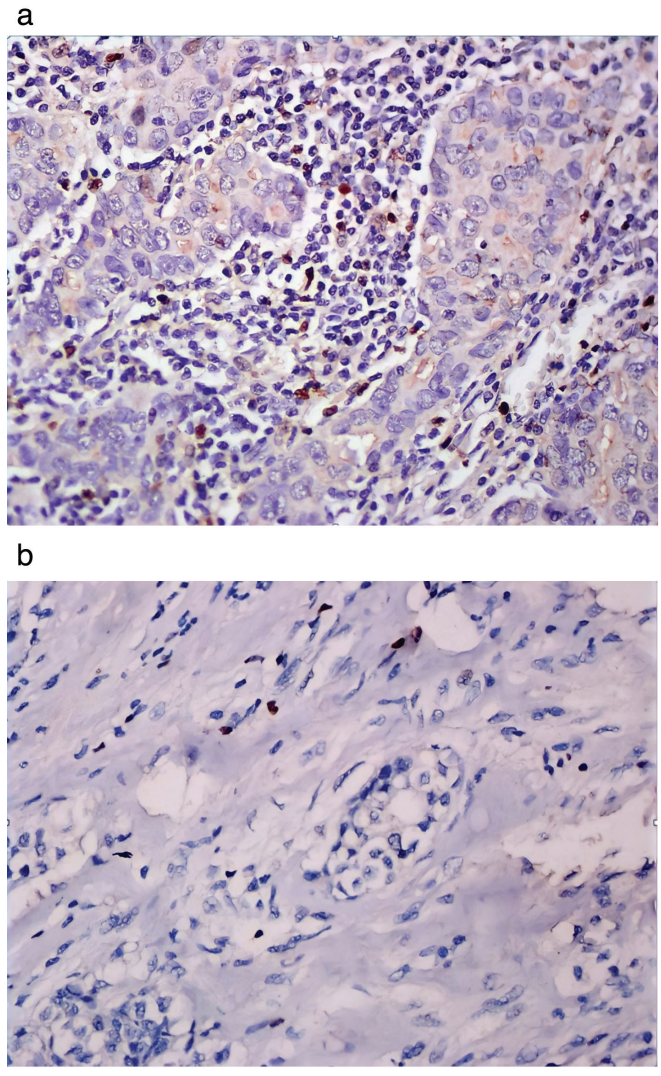


**Figure 2.** a, b) A case of invasive ductal carcinoma demonstrating infiltration with a few number of FOXP3-positive Treg cells (DAB X100)

| **Table 1. Demographic and histopathologic data** | | | |
|---|---|---|---|
| | | **n** | **%** |
| Prognosis | Survived<br>Died | 170<br>40 | 81<br>19 |
| Tumor location | Right<br>Left<br>Bilateral | 69<br>84<br>1 | 44.8<br>54.6<br>0.6 |
| Diagnosis | Invasive ductal carcinoma<br>Invasive lobular carcinoma<br>Invasive papillary carcinoma<br>Micropapillary carcinoma<br>Metaplastic carcinoma<br>Mucinous carcinoma<br>Acinar cell carcinoma<br>Tubular carcinoma<br>Ductal+lobular carcinoma | 128<br>16<br>6<br>2<br>3<br>2<br>1<br>1<br>2 | 61<br>7.6<br>2.9<br>1.0<br>1.4<br>1.0<br>0.5<br>0.5<br>1.0 |
| Grade | Grade 1<br>Grade 2<br>Grade 3 | 13<br>99<br>98 | 6.3<br>47.1<br>46.6 |
| Pathologic T stage | pT1<br>pT2<br>pT3<br>pT4 | 91<br>95<br>7<br>18 | 43.1<br>45<br>3.3<br>8.5 |
| *In situ* component | Yes | 146 | 73.4 |
| Type of *in situ* component (if any) | Comedo<br>Noncomedo<br>Mixed | 11<br>69<br>66 | 7.2<br>47.1<br>45.7 |
| Lymph node metastasis | Yes<br>No | 70<br>82 | 46<br>54 |
| Capsular invasion in the lymph node | Yes<br>No | 51<br>19 | 72.8<br>27.2 |
| Multifocality | Single<br>Multifocal | 198<br>12 | 91.5<br>8.5 |
| Nipple involvement | Yes | 15<br>43 | 25.8<br>74.2 |
| Dermal/epidermal invasion | Yes | 16 | 7.6 |
| Lymphovascular invasion | Yes | 52 | 31.9 |
| Perinueral invasion | Yes | 43 | 25.9 |

significant statistical difference between prognosis and PD-L1 positivity.

Ali et al.[8] detected PD-L1 expression using both PCR and IHC methods in a study with a large population of 3.916 cases. In their study, they found that PD-L1 was expressed in very low amounts in both tumor cells and surrounding immune cells in breast cancers. In their study, they detected PD-L1 positivity in inflammatory cells at a rate of 6%, and they observed tumoral PD-L1 positivity in only 66 (1.7%) cases. In our study, very similar data were obtained and PD-L1 was weakly expressed in only 6 (2.9%) cases, while cytoplasmic PD-L1 expression was found in inflammatory cells in 15 cases.

Muenst et al.[6] did not find any significant relationship between PD-L1 expression and molecular subtypes. Also in our study, no statistically significant difference was not observed between molecular subtypes in PD-L1-positive and-negative groups (p=0.895). Three of six cases showing PD-L1 positivity in the tumor belonged to the luminal A and the other 3 cases to the luminal B molecular subgroup, while PD-L1 positivity was not observed in the basal-and HER2-positive groups. Also, there was no significant difference between tumor grades and PD-L1 positivity (p=0.746). Although the shortest median survival was detected in breast cancer patients with tumoral PD-L1 expression, no statistically significant difference was not observed as for survival rates due to the small number of PD-L1-positive cases (p=0.660). In our study, Abcam; Ab205921-PD-L1 antibody was applied manually to microarray blocks. Karnik et al.[9] investigated

| Table 2. Immunohistochemical, and molecular findings | | | |
|---|---|---|---|
| **Parameters** | **Status** | **n** | **%** |
| ER status | Positive | 176 | 83.8 |
| PR status | Positive | 157 | 74.8 |
| c-erbB2 expression (according to ASCO/CAP 2013 criteria) | Negative or 1+<br>2+<br>3+ | 156<br>21<br>18 | 74.3<br>10<br>8.6 |
| HER2 amplification (FISH method) | Positive<br>Negative | 30<br>21 | |
| Molecular type | Luminal A<br>Luminal B<br>HER2-positive<br>Triple negative (basal-like) | 66<br>80<br>46<br>18 | 31.4<br>38.1<br>21.9<br>8.6 |
| PD-L1 expression | Positive (in inflammatory tissue)<br>Positive (in tumor)<br>Negative | 15<br>6<br>189 | 7.1<br>2.9<br>90 |
| FOXP3 expression | Tumor samples containing FOXP-positive lymphocytes | 14 | 6.7 |

the predictive value of using different antibodies in reflecting the expression. No significant difference was not found in evaluating PD-L1 expression in antibodies of different brands such as Ventana®, Dako®, and BioCare®.

In the study conducted by Tringler et al.[10] on 173 primary breast carcinoma and 246 metastatic breast carcinoma patients, they could not find a statistically significant difference between PD-L1 positivity and the stage and degree of the disease or other clinicalopathological conditions. They only observed a significant difference with negative PR receptor and HER2 status. Although these findings are similar to our study, they found PD-L1 positivity in 95% of invasive breast carcinoma cases, contrary to our study findings.

In our study, only 14 (6.7%) patients had FOXP3 + tumor-infiltrated lymphocytes. However, FOXP3 positivity was not significantly related to the prognosis or molecular typing of the tumor. Liu et al.[11] found a statistically significant relationship between FOXP3 positivity and prognosis in their study of 1.270 cases. The authors observed that infiltration with CD8 + T and FOXP3 + Treg cells were seen more frequently in basal-like breast carcinomas.

In the study of Bates et al.,[12] the number of Treg cells *in situ* carcinoma, invasive carcinoma, and normal breast tissue was compared. The greatest number of FOXP3 + Treg cells was found in invasive breast cancer, while normal breast tissue contained the least number of these cells. Higher Treg counts were associated with early relapse in cases with ductal carcinoma *in situ* and were also associated with lower survival in invasive breast cancers. In our study, considering the presence of an *in situ* component in the tumor, no statistically significant difference was not found between the presence and type of an existing *in situ* component and the presence of FOXP3-positive lymphocytes. A lot of evidence has shown that the PD-L1/PD1 molecular pathway has created a synergistic effect in the transformation of naive T cells into Treg cells in order to preserve peripheral immunity by regulating important molecular signals. Li et al.[5] reported that two determinants work synergistically by evaluating PD-L1 and FOXP3 expression in breast tumors. They associated combined PD-L1 and FOXP3 positivity with poor prognosis. However in our study, no statistically significant relationship was not found between PD-L1 expression and FOXP3 positivity. The small sample size we used in our study, the limited number of cases, and the fact that in this limited number of cases, evaluating sections that do not reflect the entire case with multiple blocks but only a small area affected our results. The factors that statistically affected survival in our study were thepresence of lymph node metastasis (p=0.006) and tumor thrombosis in dermal lymphatics (p<0.01), pathological tumor stage (p<0.01), and epidermal invasion (p=0.014). The known prognostic factors in breast carcinomas are the patient's age, tumor size, histological grade, lymph node involvement, and hormone receptor status.[13]

Evaluation of PD-L1 expression has been standardized in a few tumors such as non-small-cell lung carcinomas and malignant melanoma. However, many clinical studies have shown that PD-L1 inhibitors are safe, well-tolerated treatments with few autoimmune side effects.[14,15]

It is important to develop new treatment options for basal-like breast cancers where treatment is limited. It has been shown in many studies that PD-L1 expression increases in

basal-like breast cancers.[8] Nanda et al.[16] observed that most 111 patients in whom pembrolizumab, a PD-L1 inhibitor, was administered complied to the treatment and the drug did not cause serious side effects.

### Study Limitations

Our study was subject to certain limitations. Primarily, the study was conducted on a sample size of 210 patients. Larger sample sizes can provide more robust and reliable results, minimizing the impact of random variations. In our study, we used a microarray technique to enable IHC analysis. Instead of relying solely on tissue microarrays, future studies should consider using whole-tissue sections. This approach allows for a more comprehensive evaluation of the tumor immune microenvironment and enables analysis according to different clinicalopathological features.

## CONCLUSION

Changes in staining protocols with the use of different antibodies in PD-L1 IHC staining, lack of standardized threshold value and scoring system, and existence of different antibody-directed treatments cause problems in terms of diagnosis and treatment. Antibody-based immune treatments gain importance day by day with a better understanding of the cancer microenvironment. Therefore, studies should be conducted on PD-L1 and FOXP3 expression in many tumors, especially breast cancer, and evaluation criteria should be standardized.

### Ethics

**Ethics Committee Approval:** The study was approved by the University of Health Sciences Turkey, İzmir Tepecik Training and Research Hospital of Local Ethics Committee (protocol number: 38, date: 11.12.2017).

**Informed Consent:** Since our study is an IHC study and a study on blocks, there is no need to obtain patient consent.

**Peer-review:** Externally and internally peer-reviewed.

### Authorship Contributions

Surgical and Medical Practices: Ö.K.K., Concept: Ö.K.K., Design: Ö.K.K., G.D., C.S., Data Collection or Processing: Ö.K.K., C.S., Analysis or Interpretation: Ö.K.K., G.D., G.G., Literature Search: Ö.K.K., G.G., Writing: Ö.K.K.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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