

ORIGINAL ARTICLE

Potential prognostic value of PD-L1 and FOXP3 as predictors of relapse in breast cancer

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ABSTRACT

Background: Expression of PD-L1 detected by immunohistochemistry can represent a new hope for cancer management. The role of PD L1 in breast cancer is still unclear. Similarly, is the role of tumor-infiltrating FOXP3 +ve regulatory T (Treg) cells where literature data are conflicting. Our study aimed to evaluate the immunohistochemical expression of PD L1 and FOXP3 in breast cancer, correlate them with clinicopathological parameters as well as evaluating their relation.

Methods: This is a retrospective study carried out on 136 breast cancer specimens. Only cases with proved pathological diagnosis of infiltrating duct carcinoma of no special type (NST) were included. Tissue microarray blocks were constructed and immunostained with the polyclonal antibody for PDL1 and monoclonal antibody for FOXP3.

Results: Statistically significant correlation was found between high FOXP3 and nearly all adverse prognostic factors including; grade III tumors ($p = .003$), basal-like subtype ($p = .001$), high Ki67 ($p = .001$), negative ER status ($p = .001$), negative PR ($p = .028$), HER2 expression ($p = .04$), advanced stage ($p = .001$), and LN metastases ($p = .001$). For PDL1, only statistically significant correlation with high Ki67 ($p = .018$) and advanced stage ($p = .03$) was found. A statistically significant positive correlation was found between PD L1 and FOXP3 ($p = .001$). No statistically significant correlation was found between both PDL1 and FOXP3 in relation to disease-free survival (DFS) ($p = .054$). PDL1, age (≥ 50 years), nodal metastases were significant predictors of relapse in breast cancer.

Conclusion: The current study supports PDL1 as a predictor of relapse in breast cancer. Additionally, it highlights the synergistic role between PDL1 and FOXP3 in breast cancer microenvironment. Each can be considered as a poor prognostic marker in breast cancer. This raises a concern about the benefit of breast cancer patients from blocking of PDL1 pathway.

Key Words: Breast cancer, PDL1, FOXP3, Regulatory T cells

1. INTRODUCTION

Evasion of the immune system is one of the main mechanisms for survival of malignant cells. On the other hand, adaptive immune response against cancer is carried out, mainly, by cytotoxic T lymphocytes and type I helper T cells (CD4+). Another subpopulation of tumor infiltrating lymphocytes (TIL) is FOXP3 regulatory T (Treg) cells. The role of such cell population, whether acting against or supporting

malignant cells, isn't clear.^[1]

Antitumor immune response is also affected by immune checkpoints. These are molecules expressed on immune cells mainly T lymphocytes. These molecules can modify T cell response by delivering either costimulatory or coinhibitory signals. PD-1 is considered a coinhibitory receptor that can downregulate T-cell activity.^[2] PD-1 is activated by interacting with its ligands, either programmed death ligand-

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1 (PD-L1) or programmed death ligand-2 (PD-L2).^[3] Due to the availability of target therapy, expression of PD-L1 detected by immunohistochemistry (IHC) can represent a new hope for cancer management using monoclonal antibodies blocking the PD-1/PD-L1 pathway. Therefore, PD L1 expression was studied in various tumors and found to be a poor prognostic marker.^[4-8] However, the role of PD L1 in breast cancer is still guarded by conflicting literature data. In addition, presence of marker that can predict breast cancer recurrence is particularly valuable for guiding patient follow up.^[9] Consequently, we have carried out the current study to evaluate the IHC expression of PD L1 and FOXP3 Treg cells in breast cancer, correlate them with clinicopathological parameters as well as evaluating their relation in breast cancer.

2. MATERIALS AND METHODS

This is a retrospective study carried out on 136 formalin fixed paraffin embedded breast cancer specimens. Only cases with proved pathological diagnosis of infiltrating duct carcinoma with no special type (NST) were included in the study. Cases received neoadjuvant chemotherapy were excluded. Tissue microarray blocks were constructed. 4 μ m thick sections were immunostained (via autostainer) with rabbit monoclonal antibody for FOXP3 (CA 94588 Abcam, Cambridge; UK) and rabbit polyclonal antibody for PD L1 (YPA1637, 1: 400 dilutions, Chongqing Biopsies CO. LTD.; China). Samples were heated at 56°C, deparaffinized in xylene, and rehydrated in descending grades of alcohol. Antigen retrieval was performed by boiling in EDTA. Avidin-Biotin-Peroxidase method was used. The diaminobenzidine (DAB) chromogen system was used (Sigma-Aldrich, USA) and counterstaining was performed with Gill's hematoxylin and slide mounts in Canada balsam. Positive and negative controls were performed to verify the specificity of the primary antibody. Scoring was done by two independent pathologists blinded to prognostic data. The study was approved by our institutional ethical committee.

2.1 Scoring of FOXP3

Only nuclear reaction is considered positive. The infiltrating density of intratumoral FOXP3+ Tregs was scored semi-quantitative by estimating the percent of FOXP3 +ve cells to TILs in 10 high power fields (HPFs) then considering the median percent. It was then categorized as high or low relative to a cut-off percentage of 25%.^[10,11]

2.2 Scoring of PDL 1

The cytoplasmic &/or membranous reaction is considered positive and scored using the H score. PD-L1 expression was classified into two groups according to a cut-off H-score of

100 (0-99 = negative expression; 100-300 = positive expression).^[10]

Table 1. Clinicopathological data of studied cases

	n = 136	%
Age /years		
< 50	50	36.8
≥ 50	86	63.2
Mean ± SD	53.79 ± 12.65	
Median (range)	55.0 (28.-86.0)	
Grade		
G2	90	66.2
G3	46	33.8
Stage		
IA	4	2.9
IIB	10	7.4
IIA	44	32.4
III	40	29.4
IV	38	27.9
Molecular subtype		
Basal like	32	23.5
Her2enriched	20	14.7
Luminal A	24	17.6
Luminal B	30	22.1
Luminal HER2	30	22.1
KI 67		
HIGH	41	30.1
LOW	54	39.7
FOXP3		
low	70	51.5
high	66	48.5
ER		
-VE	52	38.2
+VE	84	61.8
PR		
-VE	72	52.9
+VE	64	47.1
HER2		
-VE	82	60.3
+VE	54	39.7
PDL1		
-VE	56	41.2
+VE	80	58.8
Relapse	N = 104	
-ve	72	69.2
+ve	32	30.8
Overall survival duration		
Mean ± SD		34.69 ± 16.17
Median (range)		34.2(3.2-92.3)
Time to relapse		
Mean ± SD		30.26 ± 15.04
Median (range)		33.3(1.7-71.9)

Table 2. Correlation between FOX P3 and clinicopathological parameters

	FOX P3		test of sig.
	Low n = 70(%)	High n = 66(%)	
age/years			
< 50	24 (34.3)	26 (39.4)	$\chi^2 = 0.38$
≥ 50	46 (65.7)	40 (60.6)	$P = .54$
Grade			
G2	58 (82.9)	32 (48.5)	$\chi^2 = 17.93$
G3	12 (17.1)	34 (51.5)	$P < .001^*$
Stage			
IA	2 (2.9)	2 (3.0)	MC $P = 0.04^*$
IIB	4 (5.7)	6 (9.1)	
IIA	30 (42.9)	14 (21.2)	
III	14 (20.0)	26 (39.4)	
IV	20 (28.6)	18 (27.3)	
Molecular subtype			
Basal like	6 (8.6)	26 (39.4)	MC
Her2enriched	6 (8.6)	14 (21.2)	$P < .001^*$
Luminal A	24 (34.3)	0 (0.0)	
Luminal B	18 (25.7)	12 (18.2)	
Luminal HER2	16 (22.9)	14 (21.2)	
KI 67			
HIGH	32 (45.7)	58 (87.9)	$\chi^2 = 26.98$
LOW	38 (54.3)	8 (12.1)	$P < .001^*$
ER			
-VE	12 (17.1)	40 (60.6)	$\chi^2 = 27.17$
+VE	58 (82.9)	26 (39.4)	$P < .001^*$
PR			
-VE	28 (40.0)	44 (66.7)	$\chi^2 = 9.69$
+VE	42 (60.0)	22 (33.3)	$P = .002^*$
HER2			
-VE	48 (68.6)	34 (51.5)	$\chi^2 = 4.13$
+VE	22 (31.4)	32 (48.5)	$P = .04^*$
LN metastases			
Negative	36 (51.4)	18 (27.3%)	MC
Positive	34 (48.6%)	48 (72.7%)	$P < .001^*$
Metastases			
Negative	50 (71.4%)	48 (72.7%)	$\chi^2 = 0.03$
Positive	20 (28.6%)	18 (27.3%)	$P = .87$

Note. MC: Monte Carlo test, χ^2 =Chi-Square test, * statistical significant

Clinical data were reviewed from patient files. Correlations were made between each IHC marker and clinicopathological parameters including age, tumor grade, stage, molecular subtype, ER status, PR status, HER 2 status, ki67, and disease-free survival (DFS) Then both markers were correlated with each other.

2.3 Statistical analysis and data interpretation

Data were fed to the computer and analyzed using IBM SPSS software package version 22.0. Qualitative data were

described using the number and percent. Significance of the obtained results was judged at the (0.05) level.

Table 3. Correlation between PDL1 and clinicopathological parameters

	PDL1		test of sig.
	-ve n = 56(%)	+ve n = 80(%)	
age/years			
< 50	20 (35.7)	30 (37.5)	$\chi^2 = 0.05$
≥ 50	36 (64.3)	50 (62.5)	$P = .83$
Grade			
G2	40 (71.4)	50 (62.5)	$\chi^2 = 1.17$
G3	16 (28.6)	30 (37.5)	$P = .28$
Stage			
IA	0 (0.0)	4 (5.0)	MC $P = .03^*$
IIB	4 (7.1)	6 (7.5)	
IIA	22 (39.3)	22 (27.5)	
III	10 (17.9)	30 (37.5)	
IV	20 (35.7)	18 (22.5)	
Molecular subtype			
Basal-like	8 (14.3)	24 (30.0)	MC $P = .15$
Her2enriched	8 (14.3)	12 (15.0)	
Luminal A	14 (25.0)	10 (12.5)	
Luminal B	14 (25.0)	16 (20.0)	
Luminal HER2	12 (21.4)	18 (22.5)	
KI 67			
HIGH	28 (50.0)	62 (77.5)	$\chi^2 = 11.13$
LOW	28 (50.0)	18 (22.5)	$P = .001^*$
ER			
-VE	16 (28.6)	36 (45.0)	$\chi^2 = 3.77$
+VE	40 (71.4)	44 (55.0)	$P = .052$
PR			
-VE	26 (46.4)	46 (57.5)	$\chi^2 = 1.62$
+VE	30 (53.6)	34 (42.5)	$P = .20$
HER2			
-VE	36 (64.3)	46 (57.5)	$\chi^2 = 0.63$
+VE	20 (35.7)	34 (42.5)	$P = .43$
LN metastases			
Negative	26 (46.4%)	28 (35.0%)	MC
Positive	30 (53.6%)	52 (65%)	$P = .24$
Metastases			
Negative	36 (64.3%)	62 (77.5%)	$\chi^2 = 2.86$
Positive	20 (35.7%)	18 (22.5%)	$P = .09$

Note. MC :Monte Carlo test, χ^2 =Chi-Square test, * statistical significant

Table 4. Correlation between FOX P3 & PD L1

PDL1	FOX P3		test of sig.
	Low n = 70(%)	High n = 66(%)	
-ve	50 (71.4)	6 (9.1)	$\chi^2 = 54.5$
+ve	20 (28.6)	60 (90.9)	$P < .001^*$

Note. χ^2 = Chi-Square test, * statistically significant

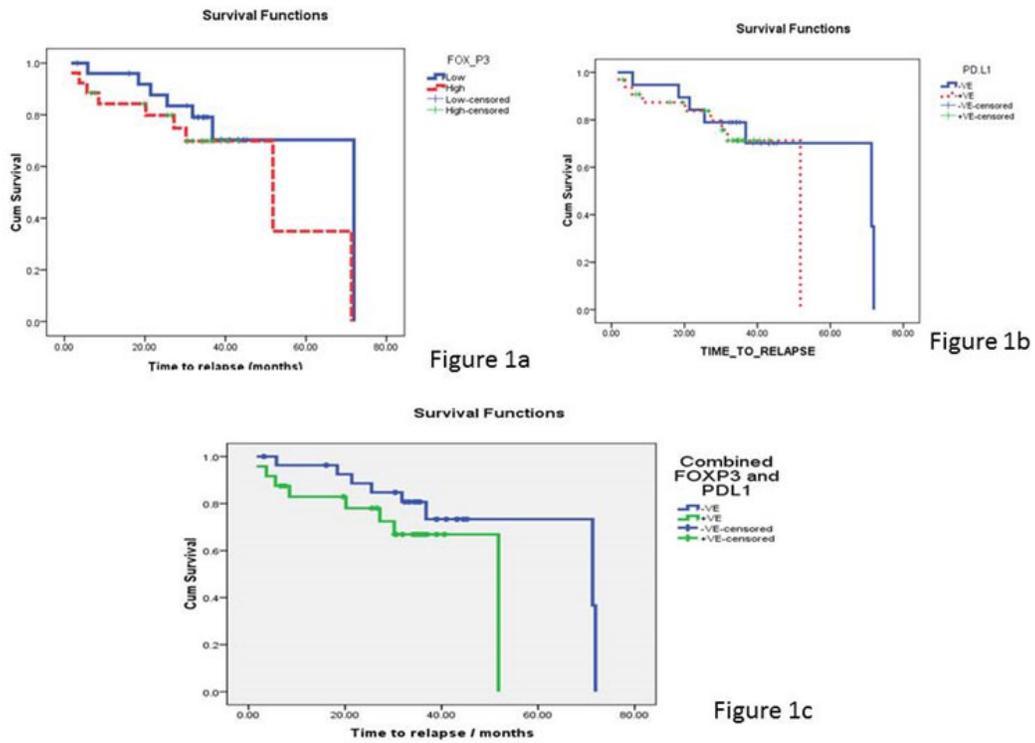


Figure 1. Kaplan-Meier survival curve for DFS depending on the expression of FOXP3 alone(1a), PDL1 alone(1b), and combined FOXP3/PDL1(1c)

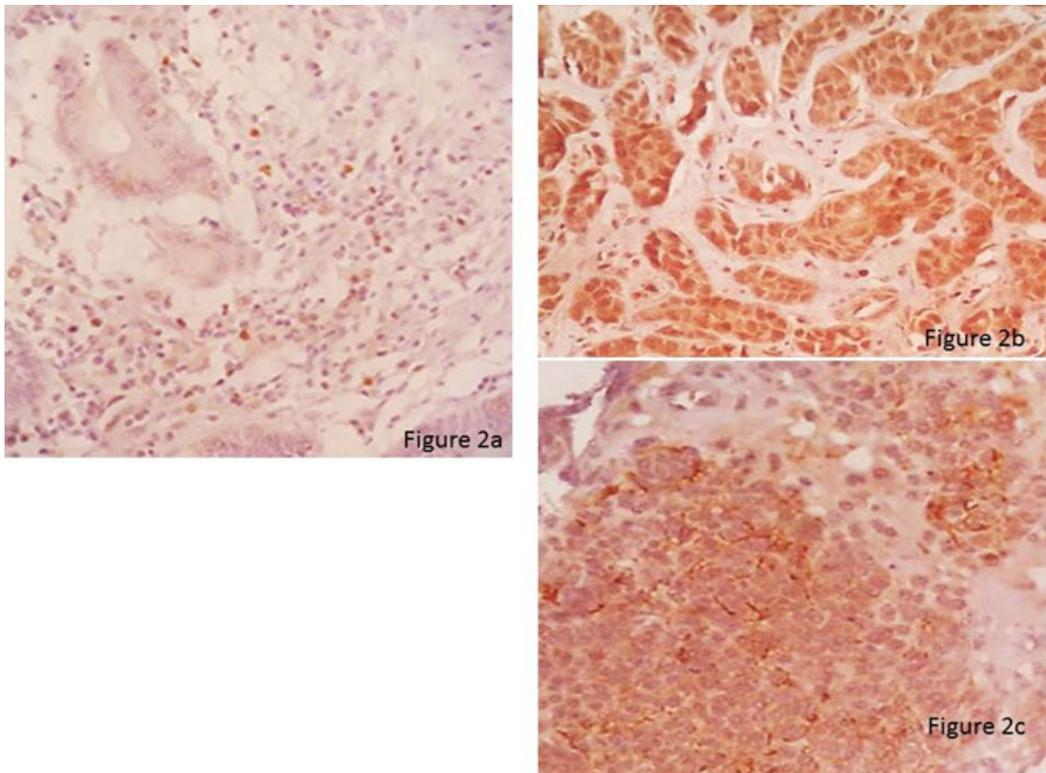


Figure 2. Nuclear expression of FOXP3 +ve intratumoral Treg cells (2ax400), cytoplasmic expression of PDL1 in tumor cells (2b, x400), both membranous and cytoplasmic expression of PDL1(2cx400)

2.4 Data analysis

2.4.1 Qualitative data

Chi-Square test for comparison of 2 or more groups

Monte Carlo test as a correction for the Chi-Square test when more than 25% of cells have count less than 5 in tables (> 2*2).

2.4.2 Kaplan-Meier test

Used to calculate disease-free survival by using log-rank χ^2 to detect the effect of risk factors affecting survival.

3. RESULTS

3.1 Clinicopathological data

The study included 136 cases of infiltrating duct carcinoma of no special type (NST). The mean age of studied cases was 53.79. Majority of cases were grade II represented by 90 cases while the remaining were grade III. Considering molecular subtype; 16 cases were basal-like, 10 cases were HER 2 enriched, 12 cases luminal A, 15 cases luminal B, and 15 cases luminal HER2. Only 4 cases were stage I, 54 cases were stage II, 40 cases were stage III, and 38 cases were stage IV. Detailed clinicopathological data were illustrated in the Table 1.

3.2 Correlation between FOXP 3 and clinicopathological parameters

70 cases revealed low expression of FOXP3 while 66 cases displayed high expression (see Figure 2a). Statistically significant correlation was found between high FOXP3 and nearly all adverse prognostic factors including; grade III tumors ($p = .003$), basal-like subtype($p = .001$), high Ki67($p = .001$), negative ER status($p = .001$), negative PR($p = .028$), HER2 expression ($p = .04$), advanced stage ($p = .001$), and LN metastases ($p = .001$). However, no significant correlation was found with age or distant metastases (see Table 2). As regard to DFS, the mean for cases with low FOXP3 (53.1 ± 3.6) was longer than that for cases with high expression (47.5 ± 4.8), but didn't reach a statistically significant level ($p = .152$) (see Figure 1a).

3.3 Correlation between PD L1 and clinicopathological parameters

56 cases were negative for PD L1 and 80 cases were positive (see Figures 2b and 2c). Only statistically significant correlation with high Ki67 ($p = .018$) and advanced stage($p = .03$) was found. On the other hand, no significant correlation was found with other clinicopathological parameters (see Table3). As for FOXP3, DFS for negative cases (57.2 ± 4.1) was longer than that for positive cases (41.9 ± 2.2) but without a statistically significant correlation ($p = .364$) (see Figure 1b).

3.4 Correlation between FOXP3 and PD L1

A statistically significant positive correlation was found between PDL1 and FOXP3($p = .001$) (see Table 4).

3.5 Correlation between DFR and combined PD L1 /FOXP3

No statistically significant correlation was found between both PDL1 and FOXP3 in relation to DFS($p = .054$) (see Figure 1c).

3.6 Multivariate regression analysis for relapse predictors

PDL1, age (≥ 50 years), nodal metastases, HER2 positive status as well as HER 2 molecular subtype were the statistically significant predictors for relapse (see Table 5).

Table 5. Multivariate analysis for detection of RELAPSE predictors

	B	p	HR	95.0% CI for HR	
				Lower	Upper
age (≥ 50 years)	-2.206	.021*	.110	.017	.714
FOX_P3	-2.246	.153	.106	.005	2.311
PDL1 (+VE)	2.338	.027*	10.357	1.313	81.698
STAGE	.643				
II B	2.472	.975	11.848	.000	1.127E68
II A	10.331	.886	30662.789	.000	5.285E65
III	-.578	.994	.561	.000	5.778E66
IV	-.568	.994	.567	.000	5.767E66
T		.525			
T(0)	-1.747	.147	.174	.016	1.846
T(2)	-1.283	.374	.277	.016	4.696
T(3)	12.612	.781	300103.399	.000	1.499E44
N		.021*			
N(1)	12.544	.694	280367.755	.000	4.284E32
N(2)	8.930	.780	7552.075	.000	1.223E31
N(3)	12.546	.695	281072.376	.000	4.730E32
M		.			
GRADE	-1.504	.164	.222	.027	1.846
ER	-4.220	.053	.015	.000	1.050
PR	1.112	.347	3.042	.300	30.836
her2(+VE)	2.687	.020*	14.684	1.526	141.286
Ki67	-1.576	.102	.207	.031	1.366
SUBTYPES		.073			
HER2 enriched	-4.013	.021*	.018	.001	.540
LUMINAL HER2	2.402	.042*	11.044	1.086	112.345

4. DISCUSSION

Breast cancer is the most common female cancer. Molecular events underlying breast cancer are still mysterious indicating more research for better patient management. Recent studies have shed lights upon the role of immune mechanisms in breast cancer with reference to immune checkpoints for which there is available target therapy. Selecting patients who may benefit from this therapy is guarded by expression of PDL1 in breast cancer as well as its prognostic role and effect on survival. Data addressing these issues in literature were conflicting.^[1] When evaluated in 136 cases of IDC (NST) in the current study, we found positive expression of PDL1 in 58.8% of cases. Our figure for PDL1 was concor-

dant to that reported by Baptista et al. (2016), higher than Muenst et al. (2014) who reported 23.4%, and much higher than Ren et al. (2018) who found it to be 6.7%.^[12-14] On the other hand, Beckers et al. (2016) reported a much higher percentage (64%) but their study included only triple-negative breast cancer cases that may explain this high difference with ours.^[15] Considering the prognostic role of PDL1, we found a significant correlation of PD L1 with two poor prognostic parameters which are high proliferative index and advanced stage. Additionally, most of the cases expressing PDL1 were basal-like subtype but didn't achieve a significant level. Although positive cases for PDL1 had shorter DFS (41.9) than negative cases (57.2), but the correlation with DFS was statistically insignificant. This may be due to a limited number of cases in our study. However, in multivariate analysis PD L1 was a statistically significant predictor for relapse. Therefore, the overall results of our study are supporting PDL1 as a poor prognostic marker in IDC. These were concordant to Muenst et al. (2014), Li et al. (2016), Li et al. (2019), and Wu et al. (2019) who considered PDL1 as poor prognostic parameter significantly correlated with reduced OS. The larger number of cases in these studies could help the achievement of statistically significant level than ours.^[10,13,16,17] In another view, Ren et al. (2018) and Noske et al (2019) found no association between PD L1 expression and clinical outcome.^[14,18] On the contrary, Schalper et al. (2014), Ali et al (2015), Baptista et al. (2016) and Bertucci et al. (2016) found up-regulation of PDL1 mRNA or protein in breast cancer to be correlated with better survival due to strong cytotoxic local immune response. High PD L1 expression can be considered a tumoral response to the strong antitumor reaction of CD8+ve T cells.^[12,19-21] When considering TILs, our study found a high level of FOXP3 positive Treg cells in 48.5% of cases. Additionally, there was a significant correlation between these cells and poor prognostic parameters as high tumor grade, basal-like subtype, high ki67, negative ER, PR HER 2 expression, and LN metastases. Similar to PDL1, DFS was shorter in cases with high FOXP3 positive TIL (47.5) than low (58.1) ones but didn't reach a significant level. Our results were matching most of those reported in the literature by Liu et al. (2011), Kim et al. (2013), Allaoui et al. (2017), and, Glajcar et al. (2019). It seems that the sup-

pressive effect of intratumoral regulatory T cells on cytotoxic CD8 positive T lymphocytes is the main mechanism helping tumor growth, invasion and metastases. This can explain the correlation of TILs with the poor prognostic parameters in breast cancer.^[22-25] On the other hand, Yeong et al. (2017) reported that a higher density of Treg cell infiltrating breast cancer stroma of triple-negative subtype is associated with favorable prognosis. This may be attributed to tumor-specific driven mechanisms affecting stromal infiltration by immune cell populations. In such a study, they found more CD8 and B cells tumoral infiltration with upregulation of genes involved in immune functions. Such specific mechanisms need a further declaration.^[26] Our study was one of few studies addressing the correlation between tumor expression of PDL1 and intratumoral FOXP3+ve Treg cells. We found a significant positive association between PDL1 expression by tumor and FOXP3+ve TILs. Our results were concordant to Li et al. (2016). On the other hand, Park, et al. (2016) found no association between both of them. This discrepancy may be because Park et al. (2016) conducted their study on cases of early-stage breast cancer while both of PDL1 and FOXP3 are more commonly expressed in advanced stages^[10,27] From the current study we conclude that PDL1 can be considered as a predictor for breast cancer recurrence. Additionally, we support the synergistic role between PDL1 and FOXP3 in breast cancer microenvironment. Each can be considered as a poor prognostic marker in breast cancer. In this way our study may help to fill the gap about the prognostic and predictive value of PDL1 and FOXP3 for breast cancer patients. This highlights the benefit of such patients from blocking of PDL1 pathway. These results may be validated on wider scale studies addressing different histotypes of breast cancer. We had some limitations including a small number of cases due to limited archival follow up data, as well as the use of TMA. However, we tried to overcome this by a sampling of 3 cores from each case. Additionally, we used IHC only without correlation with mRNA expression but we aimed to detect the value of IHC alone being more economical and more widely used in our institution.

CONFLICTS OF INTEREST DISCLOSURE

The author declares no conflict of interest

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