RESEARCH ARTICLE

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Expression of PD-L1 in Early-Stage Invasive Breast Carcinoma and Its Relation to Tumor-Infiltrating Lymphocytes

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Abstract

Objectives: Immunotherapeutic targets became one of the promising approaches in breast cancer (BC), especially in advanced stage triple-negative subtype (TNBC). However, the role of programmed cell death ligand 1 (PD-L1) targeting in other BC subtypes, especially in early-stage carcinoma is less explored. We aimed in this study to investigate the prevalence of PD-L1 in early-stage invasive BC of different molecular subtypes and to elucidate its relation to tumor-infiltrating lymphocytes (TILS) density (cytotoxic and regulatory T-cells), established clinicopathological factors and patients' outcome. **Material and Methods:** One hundred and nine cases of early-stage BC were enrolled in our study. Cases were classified into five molecular subtypes according to the Immunohistochemical data. PD-L1, FOXP3 and CD8 immunostaining were analyzed for all studied cases. PD-L1 expression was correlated with CD8+ cytotoxic T-cells, FOXP3+ regulatory T-cells, histopathologic parameters, BC molecular subtypes, 7-years disease-free survival (DFS) and overall survival (OS). **Results:** PD-L1 was expressed in 11% of the studied early-stage BC cases. It showed a significant correlation with high tumor grade (p= <0.001), development of metastasis (p=0.037), high FOXP3+ T-cell density (p= <0.001) and low CD8+ T-cells density (p= <0.001). PD-L1 expression was higher in TNBC (16.1%), followed by HER2/neu-enriched group (14.3%). All luminal A cases showed negative PD-L1 expression. PD-L1 was found to be an independent prognostic factor for patients' survival (DFS; p=0.031 and OS: p=0.04). **Conclusion:** Although the impact of PD-L1 on early-stage BC outcomes had not been clearly established, our results indicated that PD-L1 is a negative prognostic marker in early settings. PD-L1 can serve as a new therapeutic target for patients with high-grade early-stage breast carcinoma.

Keywords: PD-L1- early-stage- breast cancer- FOXP3- CD8

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Introduction

A large proportion of early-stage breast cancer (BC) patients (stage I and II) achieves improvement with the standard treatment approach, however about 20% of Her2-positive cases relapse or develop an advanced disease (Lambertini et al., 2017). Moreover, complete pathologic curative rate is not achieved in up to 60% of TNBC following neoadjuvant chemotherapy. As for hormone receptor-positive cases, recurrence can occur even twenty years following treatment (Pan et al., 2017). For these aspects, it is recommended to modulate the current treatment strategy for patients with early-stage BC, those having a higher chance of complete curable rate.

Programmed Death Ligand 1 (PD-L1) is a biomarker that's often over-expressed in some tumors like lung, bladder, colorectal and renal cancer. Through its binding to PD-1 (Programmed Cell Death 1); downregulation of the immune responses occurs via CD8+T cells exhaustion or even through its conversion into regulatory T (Treg) cells (Helmy et al., 2020). Treg cells express FOXP3 which has an important role in cancer immunosuppression (Azhar and Aisyi, 2021).

While triple negative breast cancer subtype (TNBC) is rich in tumor infiltrated lymphocytes (TILS) with activation of PD-1/PD-L1 pathway, the use of immunotherapy together with chemotherapy has been approved as a first-line therapy for the advanced or metastatic settings (Franzoi et al., 2021). So far, a limited data is available for the role of immune checkpoints (IC) modulation and the impact of PD-L1 expression in early-stage BC other than TNBC.

Notably, it was found that BC loses its immunologic response over time through the decrease in TILS. Studies found that there is a decrease in immunologic signature in metastatic BC as compared to the primary tumor (Szekely

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et al., 2018; Hutchinson et al., 2020). This could explain the reported data about the more effective role for immune checkpoint blockage of PD-L1 in early stage TNBC as compared to that in advanced cases (Schmid at al., 2018; Schmid et al., 2020).

TILS is an important biomarker in predicting the response of BC to immunotherapy alone or in combination with chemotherapy (Karn et al., 2020). In Her2-positive BC, high base line TILS (≥60%) was observed in about 20% of early stage and associated with the achievement of pCR. The adding of upfront anti-PD-L1 to the treatment strategy to these patients was suggested (Solinas et al., 2017). Although hormone-receptor-positive BC usually express low levels of TILS and PD-L1 (Denkert et al., 2018 and Sobral-Leite et al., 2018), these tumors are heterogenous where some of which can exhibit a high level of stromal lymphocytes (Haricharan et al., 2014). The relationship between PD-L1 and TILS profiles remains not well established in different BC subtypes, particularly the hormone-positive cases.

The present study aimed to investigate the impact of PD-L1 expression on the outcome of early-stage BC of different molecular subtypes, and its correlation with different clinicopathologic features. Among different types of TILs, we aimed to investigate the relation of both FOXP3+ and CD8+ lymphocyte density to PD-L1 expression in early-stage BC. Such data would support the usefulness of these biomarkers in early settings of BC.

Materials and Methods

This retrospective study was carried out on one hundred and nine cases of women patients diagnosed as early-stage invasive breast carcinoma (Stages IA, IB, IIA and IIB) according to TNM staging system. To avoid false results as a consequence of using biopsy material with the heterogeneous TILs and PD-L1 expression, we used the whole tumor tissue sections of mastectomy/conservative breast surgery (CBS). Cases were retrieved from the Pathology Department, National Cancer Institute (NCI), Cairo University, throughout the period from January 2012 to December 2015. Follow up time was up to 100.6 months with a median period of 71 months. None of the included patients received neoadjuvant chemotherapy or immunotherapy.

Microscopic review of the cases for confirming the diagnosis and tumor grading were assessed according to World Health Organization (WHO) Classification of Breast Tumors, Fifth Edition, 2019 (Rakha et al., 2019). Pathologic stage was determined by examining the excised specimens, according to tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (AJCC), 8th edition (Giuliano et al., 2017).

Data of ER, PR, and Her2 were all reviewed and reported according to the updated American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) 2018 guidelines (Wolff et al., 2018; Allison et al., 2020)

Immunohistochemistry (IHC)

Sections of 4 μm were cut from the paraffin-embedded tissues and placed onto positive charged slides. Standard immunostaining was done using BenchMark ULTRA (Ventana) autostainer according to the manufacturer's instruction. Primary monoclonal antibodies (ready-to-use) were used as follows: Rabbit monoclonal antibodies against PD-L1 (RBT-PDL1), Cat No (BSB 2651), rabbit monoclonal antibodies for FOXP3 (EP 340), Cat No (BSB 2924), rabbit monoclonal antibodies (Roche) for CD8 (SP57), Cat no790-4460. Tissue sections from normal tonsil were used as a positive control for FOXP3 and CD8, while normal human placental tissue was used as a positive control for PD-L1.

PD-L1 IHC analysis

Using the high-power field (x400); the whole slide was examined to detect and calculate the percentage of PD-L1 positivity. In our cases, PD-L1 expression was found either in tumor-infiltrating immune cells (IC) alone or in both tumor-infiltrating immune cells and invasive tumor cells (TCIC). None of our cases revealed positive expression of PD-L1 in tumor cells only.

The percentage of PD-L1 expression in TCIC was calculated as the number of those cells showing PD-L1 staining (membranous staining for invasive tumor cells and any staining for immune cells) divided by the total number of invasive tumor cells. While for cases with PD-L1 expression in IC only, the percent was assessed as the proportion of tumor area occupied by PD-L1-positive immune cells of any intensity in any cell compartment. Percentage 1% or greater was considered positive (Guo et al., 2020).

FOXP3 assessment by IHC

The percentage of FOXP3 positive cells with nuclear staining expressed in both tumor cells and stromal lymphocytes [Intra and peritumoral area] was calculated manually by counting the cells in 10 high-power fields (x400). Scoring of FOXP3+ cells was as follows: 0 (no expression), 1+ (1-25% positive cells), 2+ (26-50% positive cells), 3+ (51-100 positive cells).

According to Takenaka and colleagues' study scoring protocol, scores of 0 and 1+ were interpreted as negative (absent or low infiltration), while 2+ and 3+ were positive (high infiltration). Nuclear expression of FOXP3 expression in ≥30% of TC was defined as positive, while <30% is considered negative. (Takenaka et al., 2013).

CD8+ TILs density assessment by IHC

The percentage of CD8+ T-lymphocytes was calculated by choosing 5 fields with the highest TILs infiltration, then the mean of the 5 fields was used to express the density of CD8+ TILs (percent) (Wang et al., 2017).

To estimate the best cut off point of CD8 TILs, a Receiver Operating Characteristics (ROC) curve was used. The cut off value was calculated as 20% (sensitivity 70%, specificity 82%). In all, ≤ 20% was defined as a low- density infiltration and > 20% as a high-density infiltration.

Statistical Methods

IBM SPSS advanced statistics (Statistical Package

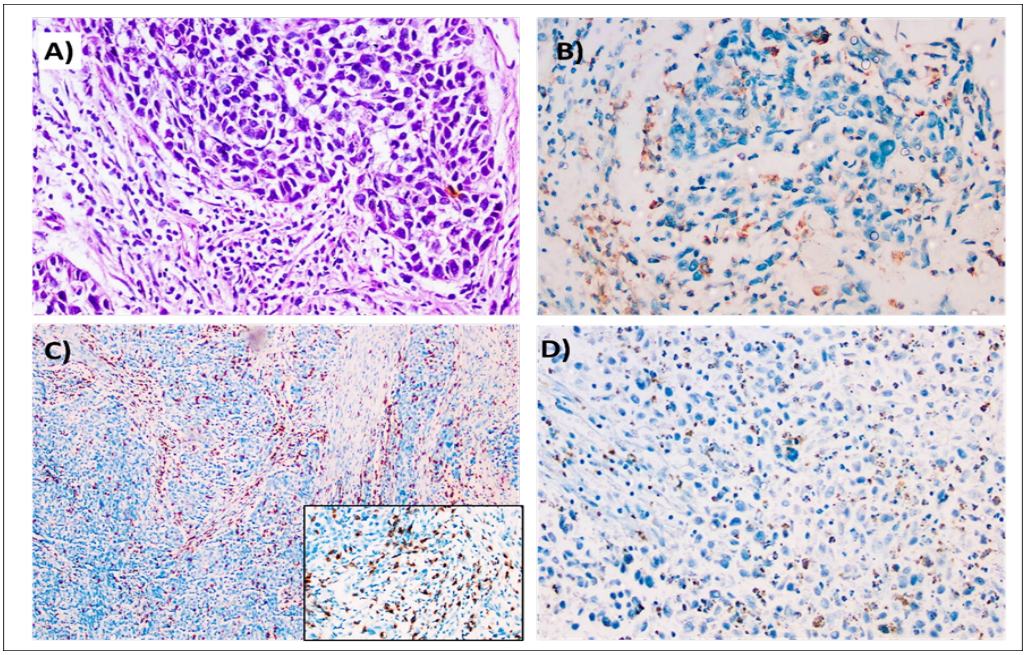

Figure 2. Another Example of Immunohistochemical Staining Results. A) Hematoxylin and Eosin image of a case high grade invasive duct carcinoma (x400). B) Immunostaining image of PD-L1 positive membranous expression in tumor cells (x400). C) Immunostaining image showing positive nuclear FOXP3 staining in TILs (x200) & inset, x400). D) Immunostaining image showing membranous expression of CD8 in peritumoral lymphocytes with low density (x400).

for Social Sciences), version 24, was used to analyze the data (SPSS Inc., Chicago, IL). The standard chi-squared (Fisher's exact) test was used to determine the relationship between categorical variables. Using a logistic regression model, multivariate analysis was performed on variables that were statistically significant on a univariate level to identify independent prognostic factors and to eliminate the effect of confounders. A Cox regression model was used to calculate the hazard ratio (HR) and its 95 percent confidence interval, and survival curves were plotted using Kaplan–Meier estimates. For survival endpoints, DFS and OS were used. A p-value of 0.05 or less was considered statistically significant.

Results

Clinicopathological findings

Detailed clinical and pathologic features are shown in Table 1. Follow up time was up to 100.6 months with a median period of 71 months (range, 49.7–100.6 months). For the whole group, 7-years disease free (DFS) survival was 68.7%and the overall survival (OS) was 83.2%.

Based on immunohistochemical criteria for defining breast cancer molecular subtypes (Rakha et al., 2019);

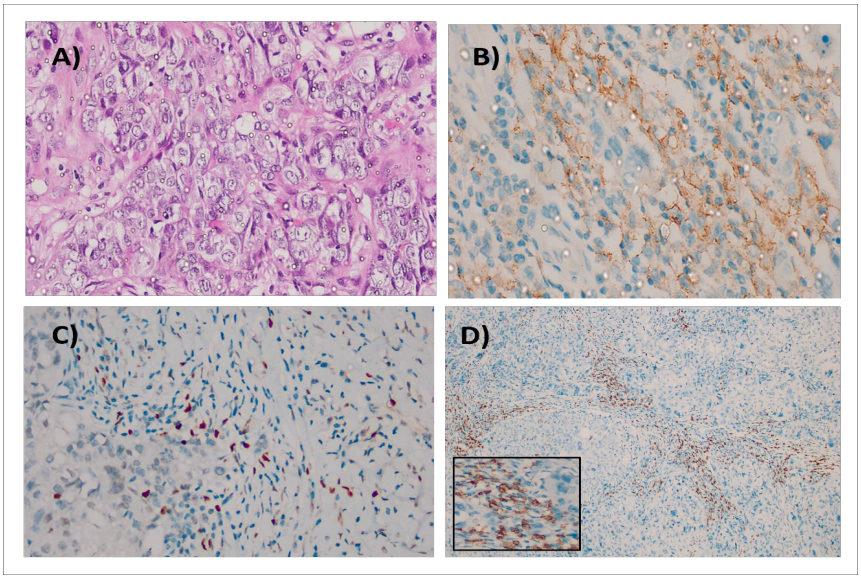

Figure 1. Example of Immunohistochemical Staining Results. A) Hematoxylin and Eosin image of a case high grade invasive duct carcinoma (x400). B) Immunostaining image of PD-L1 positive membranous expression in tumor cells (x400). C) Immunostaining image showing positive nuclear FOXP3 staining in TILs (x400). D) Immunostaining image showing membranous expression of CD8 in peritumoral lymphocytes with low density (x200) & inset, x400).

Table 1. Clinicopathologic Characteristics of the Studied Cases (no. =109).

Patients' characteristics	no. (%) Total=109
Mean age	50±10.4 years [range 25-84]
≤ 50 years	54 (49.5)
>50 years	55 (50.5)
Histopathologic type	
Invasive duct carcinoma	92 (72.5)
Invasive lobular carcinoma	3 (2.8)
Mixed invasive duct & invasive lobular carcinoma	6 (5.5)
Other BC subtypes	21 (19.2)
Pathologic T	
T1	35 (32.1)
T2	70 (64.2)
T3	4 (3.7)
Tumor grade	
I	8 (7.3)
II	77 (70.6)
III	24 (22.0)
Pathologic N	
N0	57 (52.3)
N1	52 (47.7)
Tumor stage	
IA	22 (20.2)
IIA	44 (40.4)
IIB	43 (39.4)
Metastatic sites	
Bone	8 (38)
Lung	3 (14.3)
Liver	1 (4.8)
Brain	1 (4.8)
Supraclavicular L. nodes	1 (4.8)
Multiple sites (bone, lung & liver)	6 (28.6)
Surgical procedure	
Modified radical mastectomy	24 (22)
BCS & LN excision (SLNB/ALND)	85 (78)
Chemotherapy treatment	
Adjuvant chemotherapy	101 (92.7)
No chemotherapy	8 (7.3)
Hormonal therapy	
Yes	71 (65.1)
No	38 (34.9)
Radiotherapy	
Yes	97 (89)
No	12 (11)
ER status	
Positive	70 (64.2)
Negative	39 (35.8)
PR status	
Positive	69 (63.3)
Negative	40 (36.7)
HER2/neu	
Positive	21 (19.3)
Negative	88 (80.7)
KI-67 LI (no.= 71 cases)	
≥20%	58 (81.7)
<20%	13 (18.3)
Molecular subtypes	
Luminal A	13 (11.9)
Luminal B-Her2 negative	44 (40.4)
Luminal B-Her2 positive	14 (12.8)
Her2/neu-enriched	7 (6.4)
TNBC	31 (28.4)

BCS breast conserving surgery, SLNB sentinel lymph node biopsy, ALND axillary lymph node dissectio

our cases were classified into five molecular subgroups as follows: Luminal A (ER+, PR+, HER2- and Ki67% < 20%) = group 1 (13 cases; 11.9%), Luminal B-Her2 negative (ER+, PR- or low, HER2- and Ki67% ≥ 20%) = group 2 (44 cases; 40.4%), Luminal B-Her2 positive (ER+, HER2+, PR any and Ki67 any) = group 3 (14 cases; 12.8%), HER2-enriched (ER-, PR- and HER2+) = group 4 (7 cases; 6.4%) and TNBC = group 5 (31 cases; 28.4%).

Expression of PD-L1 and its correlation with clinicopathologic characteristics

Twelve cases (11%) expressed PDL1. Out of which, seven cases showed positive expression in both TCs and TILs, while the remaining showed expression only in TILs.

We found a strong association between PD-L1 protein expression with high tumor grade (p value = <0.001) and with the development of distant metastasis on follow up (P=0.037) There was a trend towards significance in the relation between PD-L1 and both patients' age and tumor stage. Within age group ≤ 50 years; PD-L1 expressed in 9 out of 54 cases (16.7 %). While in patients > 50 years (55 cases), only 3 cases expressed PD-L1 (5.5 %) (p=0.062). Cases with stage II showed PD-L1 expression in 12 out of 87 cases, 13.8 %, while no expression reported among those of stage I [p=0.065] (Table 2). We also noticed that PD-L1 is expressed more in cases with tumor size > 2.7 cm (8/54 cases, 14.8%) as compared to those with tumor size ≤ 2.7 cm (4/55, 7.3%). However, this finding was not statistically significant (p= 0.208). No significant correlation found with other variables.

Multivariate analysis revealed that tumor grade is an independent prognostic factor affecting PD-L1 expression (p=0.025; OR=4.105, 95%CI 1.19 to 14.15).

As regard the relation between PD-L1 expression and different BC molecular subtypes; TNBC group showed the highest expression (5/31 TNBC cases, 16.1%), followed

Table 2. The Relation between PD-L1, Clinico-pathological Variables, TILs and Breast Cancer Molecular Subtypes

Parameters			PDL1		Total	p value
			Negative (n=97)	Positive (n=12)		
Age (years)	<=50	no	45	9	54	0.062
		%	83.30%	16.70%	100.00%	
	>50	no	52	3	55	
		%	94.50%	5.50%	100.00%	
Tumor size (max.) (cm)	<=2.7	no	51	4	55	0.208
		%	92.70%	7.30%	100.00%	
	>2.7	no	46	8	54	
		%	85.20%	14.80%	100.00%	
Tumor grade	I	no	8	0	8	<0.001
		%	100.00%	0.00%	100.00%	
	II	no	75	2	77	
		%	97.40%	2.60%	100.00%	
	III	no	14	10	24	
		%	58.30%	41.70%	100.00%	
Nodal status	Negative	no	50	7	57	0.657
		%	87.70%	12.30%	100.00%	
	Positive	no	47	5	52	
		%	90.40%	9.60%	100.00%	
TN Staging						
T	1	no	34	1	35	*
		%	97.10%	2.90%	100.00%	
	2	no	60	10	70	
		%	85.70%	14.30%	100.00%	
	3	no	3	1	4	
		%	75.00%	25.00%	100.00%	
N	0	no	50	7	57	0.657
		%	87.70%	12.30%	100.00%	
	1	no	47	5	52	
		%	90.40%	9.60%	100.00%	
Stage	IA	no	22	0	22	0.118
		%	100.00%	0.00%	100.00%	
	IIA	no	37	7	44	
		%	84.10%	15.90%	100.00%	
	IIB	no	38	5	43	
		%	88.40%	11.60%	100.00%	
Stage	I	no	22	0	22	0.065
		%	100.00%	0.00%	100.00%	
	II	no	75	12	87	
		%	86.20%	13.80%	100.00%	
Histopathology	Invasive duct carcinoma	no	71	8	79	0.888
		%	89.90%	10.10%	100.00%	
	Other types	no	26	4	30	
		%	86.70%	13.30%	100.00%	
ER status	Negative	no	33	6	39	0.276
		%	84.60%	15.40%	100.00%	
	Positive	no	64	6	70	
		%	91.40%	8.60%	100.00%	

Table 2. Continued

Parameters			PDL1			
			Negative (n=97)	Positive (n=12)		
PR status	Negative	no	34	6	40	0.311
		%	85.00%	15.00%	100.00%	
	Positive	no	63	6	69	
		%	91.30%	8.70%	100.00%	
HER2 status	Negative	no	78	10	88	0.809
		%	88.60%	11.40%	100.00%	
	Positive	no	19	2	21	
		%	90.50%	9.50%	100.00%	
Local recurrence	No	no	93	11	104	0.448
		%	89.40%	10.60%	100.00%	
	Yes	no	4	1	5	
		%	80.00%	20.00%	100.00%	
Metastasis	No	no	81	7	88	0.037
		%	92.00%	8.00%	100.00%	
	Yes	no	16	5	21	
		%	76.20%	23.80%	100.00%	
CD8 (%)	Low <=20	no	26	12	38	<0.001
		%	68.40%	31.60%	100.00%	
	High >20	no	71	0	71	
		%	100.00%	0.00%	100.00%	
FOXP3	Negative	no	85	0	85	<0.001
		%	100.00%	0.00%	100.00%	
	Positive	no	12	12	24	
		%	50.00%	50.00%	100.00%	
Molecular Subtypes	Luminal A	no	13	0	13	0.603
		%	100.00%	0.00%	100.00%	
	Luminal B-Her2 negative	no	39	5	44	
		%	88.60%	11.40%	100.00%	
	Luminal B-Her2 positive	no	13	1	14	
		%	92.90%	7.10%	100.00%	
	Her2-enriched	no	6	1	7	
		%	85.70%	14.30%	100.00%	
	Triple negative	no	26	5	31	
		%	93.90%	16.10%	100.00%	

*, p value cannot be assessed due to small number within strata.

by HER2/neu-enriched group (1/7 cases, 14.3%), then luminal B-Her2 negative (5/44 cases, 11.4%) and luminal B-Her2 positive (1/14 cases, 7.1%). Luminal A group didn't express PD-L1 at all. However, this difference was statistically non-significant (p value = 0.603).

FOXP3 and CD8 IHC results and their relation to PD-L1 expression and other clinicopathologic characteristics

In the present cohort study, twenty-four cases (22%) showed positive reaction to FOXP3 in TILs (including four cases showing also nuclear reaction in tumor cells), while most of the cases (71 cases; 65.1%) showed high CD8 infiltration. We found a significant inverse relation between FOXP3 and CD8 density (p value=0.025).

Our findings revealed a strong association between PD-L1 expression and type of TILs. PD-L1 showed marked expression in tumors expressing high FOXP3+ TILs and low CD8 infiltrate. On the other hand, PD-L1 is negative in tumors with negative FOXP3 TILs and high CD8+ infiltrate (p value <0.001) (Table 2). Representative examples of BC cases with expression of PD-L1, CD8 and FOXP3 are shown in Figures 1 and 2.

FOXP3 showed a strong association with higher tumor grade (p = <0.001). No significant correlation found with other studied variables (Table 3). Among different clinicopathologic features, high CD8+ stromal lymphocyte density showed a strong inverse association with both tumor size (p value = 0.038) and tumor grade (p= 0.02) (Table 4).

Table 3. The Relation between FOXP3, Clinico-Pathological Variables and Breast Cancer Molecular Subtypes

Parameters			FOXP3			
			Negative (n=85)	Positive (n=24)	Total	p value
Age (years)	<=50	no	41	13	54	0.608
		%	75.9%	24.1%	100.0%	
	>50	no	44	11	55	
		%	80.0%	20.0%	100.0%	
Tumor size (max.) (cm)	<=2.7	no	45	10	55	0.329
		%	81.8%	18.2%	100.0%	
	>2.7	no	40	14	54	
		%	74.1%	25.9%	100.0%	
Tumor grade	I	no	8	0	8	<0.001
		%	100.0%	0.0%	100.0%	
	II	no	66	11	77	
		%	85.7%	14.3%	100.0%	
	III	no	11	13	24	
		%	45.8%	54.2%	100.0%	
Nodal status	Negative	no	43	14	57	0.502
		%	75.4%	24.6%	100.0%	
	Positive	no	42	10	52	
		%	80.8%	19.2%	100.0%	
TN Staging						
T	1	no	31	4	35	0.083
		%	88.6%	11.4%	100.0%	
	2	no	52	18	70	
		%	74.3%	25.7%	100.0%	
	3	no	2	2	4	
		%	50.0%	50.0%	100.0%	
N	0	no	43	14	57	0.502
		%	75.4%	24.6%	100.0%	
	1	no	42	10	52	
		%	80.8%	19.2%	100.0%	
Stage	IA	no	20	2	22	0.247
		%	90.9%	9.1%	100.0%	
	IIA	no	32	12	44	
		%	72.7%	27.3%	100.0%	
	IIB	no	33	10	43	
		%	76.7%	23.3%	100.0%	
Stage	I	no	20	2	22	0.101
		%	90.9%	9.1%	100.0%	
	II	no	65	22	87	
		%	74.7%	25.3%	100.0%	
Histopathology	Invasive duct carcinoma	no	63	16	79	0.374
		%	79.7%	20.3%	100.0%	
	Other types	no	22	8	30	
		%	73.3%	26.7%	100.0%	
ER status	Negative	no	30	9	39	0.842
		%	76.9%	23.1%	100.0%	
	Positive	no	55	15	70	
		%	78.6%	21.4%	100.0%	

Table 3. Continued

Parameters			FOXP3			
			Negative (n=85)	Positive (n=24)	Total	p value
PR status	Negative	no	31	9	40	0.926
		%	77.5%	22.5%	100.0%	
	Positive	no	54	15	69	
		%	78.3%	21.7%	100.0%	
HER2 status	Negative	no	71	17	88	0.164
		%	80.7%	19.3%	100.0%	
	Positive	no	14	7	21	
		%	66.7%	33.3%	100.0%	
Local recurrence	No	no	81	23	104	1.000
		%	77.9%	22.1%	100.0%	
	Yes	no	4	1	5	
		%	80.0%	20.0%	100.0%	
Metastasis	No	no	71	17	88	0.164
		%	80.7%	19.3%	100.0%	
	Yes	no	14	7	21	
		%	66.7%	33.3%	100.0%	
Molecular Subtypes	Luminal A	no	11	2	13	0.286
		%	84.6%	15.4%	100.0%	
	Luminal B-Her2 negative	no	37	7	44	
		%	84.1%	15.9%	100.0%	
	Luminal B-Her2 positive	no	8	6	14	
		%	57.1%	42.9%	100.0%	
	Her2-enriched	no	6	1	7	
		%	85.7%	14.3%	100.0%	
	Triple negative	no	23	8	31	
		%	74.2%	25.8%	100.0%	

Association of PD-L1 with OS and DFS

PD-L1 expression showed a significant association with DFS. Cases experienced negative PD-L1 had a better DFS than PD-L1 positive cases (70.6% versus 50%, respectively (P=0.007) (Figure 3A).

Multivariate analysis revealed that PD-L1 and PR status were the only two independent factors affecting disease-free survival (P=0.031; HR=2.798, 95% CI 1.100 to 7.120, and P= 0.047; HR=2.270. 95% CI 1.1010-5.100, respectively).

It was observed that two main factors affected OS of the studied cases; the difference in BC molecular subtypes and the expression of PD-L1.

BC molecular subtypes associated significantly with OS (P=0.045) with the lowest O.S found in TNBC group (70.2%) followed by HER2/neu-enriched group (85.7%) then luminal group (88.7%) that included luminal B-Her2 negative group (86.3%), luminal B-HER2/neu positive group (92.9%) and luminal A group (92.3%).

Additionally, cases with negative PD-L1 experienced better OS than those with positive PD-L1 (P=0.008) (Figure 3B). PDL1 proved to be an independent factor affecting OS (P=0.040, HR=3.01, 95% CI 1.054-8.602).

Discussion

The success in ER+/PR+ and HER2 targeted therapies has shifted the researchers' interest in PD-L1 into the triple negative disease. However, PD-L1 targeted therapies may be also important for those developed resistance to the current hormone and HER2 directed therapies (Sanilmanejad et al., 2019). Therefore, we investigated the role of PD-L1 in early-stage BC of different molecular subtypes and its correlation with clinicopathologic parameters and TILs (CD8+ and FOXP3+ T cells).

Different studies have reported different results about status of PDL-1 in BC. This may be related to the different antibody clones and different approved assays with unequal sensitivity and reproducibility. In our study, the expression of PD-L1 in both tumor and immune cells was evaluated. Only 11% of our cases expressed PD-L1. In concordance with our results, Kitano et al., (2017) and Guo et al., (2020) studied the frequency of PD-L1 expression in early-stage breast carcinomas and reported PD-L1 rates of 10% and 13%, respectively. Whereas, in Berckelaer and colleagues' study, PD-L1 in tumor cells was very rare (1.9%), while in TILs the expression was much higher (43%) (Van Berckelaer at al., 2019).

Table 4. The Relation between CD8, Clinicopathological Variables and Breast Cancer Molecular Subtypes

Parameters			CD8			
			Low (n=38)	High (n=71)	Total	p value
Age (years)	<=50	no	19	35	54	0.944
		%	35.2%	64.8%	100.0%	
	>50	no	19	36	55	
		%	34.5%	65.5%	100.0%	
Tumor size (max.) (cm)	<=2.7	no	14	41	55	0.038
		%	25.5%	74.5%	100.0%	
	>2.7	no	24	30	54	
		%	44.4%	55.6%	100.0%	
Tumor grade	I	no	3	5	8	0.016
		%	37.5%	62.5%	100.0%	
	II	no	21	56	77	
		%	27.3%	72.7%	100.0%	
	III	no	14	10	24	
		%	58.3%	41.7%	100.0%	
Nodal status	Negative	no	20	37	57	0.959
		%	35.1%	64.9%	100.0%	
	Positive	no	18	34	52	
		%	34.6%	65.4%	100.0%	
TN Staging						
T	1	no	7	28	35	*
		%	20.0%	80.0%	100.0%	
	2	no	30	40	70	
		%	42.9%	57.1%	100.0%	
	3	no	1	3	4	
		%	25.0%	75.0%	100.0%	
N	0	no	20	37	57	0.959
		%	35.1%	64.9%	100.0%	
	1	no	18	34	52	
		%	34.6%	65.4%	100.0%	
Stage	IA	no	5	17	22	0.390
		%	22.7%	77.3%	100.0%	
	IIA	no	16	28	44	
		%	36.4%	63.6%	100.0%	
	IIB	no	17	26	43	
		%	39.5%	60.5%	100.0%	
Stage	I	no	5	17	22	0.181
		%	22.7%	77.3%	100.0%	
	II	no	33	54	87	
		%	37.9%	62.1%	100.0%	
Histopathology	Invasive duct carcinoma	no	25	54	79	0.336
		%	31.6%	68.4%	100.0%	
	Other types	no	13	17	30	
		%	43.30%	56.70%	100.0%	
ER status	Negative	no	15	24	39	0.556
		%	38.5%	61.5%	100.0%	
	Positive	no	23	47	70	
		%	32.9%	67.1%	100.0%	

Table 4. Continued

Parameters			CD8			
			Low (n=38)	High (n=71)	Total	p value
Parameters			CD8			
			Low (n=38)	High (n=71)	Total	p value
PR status	Negative	no	14	26	40	0.982
		%	35.0%	65.0%	100.0%	
	Positive	no	24	45	69	
		%	34.8%	65.2%	100.0%	
HER2 status	Negative	no	31	57	88	0.870
		%	35.2%	64.8%	100.0%	
	Positive	no	7	14	21	
		%	33.3%	66.7%	100.0%	
Local recurrence	No	no	37	67	104	0.656
		%	35.6%	64.4%	100.0%	
	Yes	no	1	4	5	
		%	20.0%	80.0%	100.0%	
Metastasis	No	no	30	58	88	0.729
		%	34.1%	65.9%	100.0%	
	Yes	no	8	13	21	
		%	38.1%	61.9%	100.0%	
Molecular Subtypes	Luminal A	no	2	11	13	0.615
		%	15.4%	84.6%	100.0%	
	Luminal B-Her2 negative	no	17	27	44	
		%	38.6%	61.4%	100.0%	
	Luminal B-Her2 positive	no	5	9	14	
		%	35.7%	64.3%	100.0%	
	Her2-enriched	no	2	5	7	
		%	28.6%	71.4%	100.0%	
	Triple negative	no	12	19	31	
		%	38.7%	61.3%	100.0%	

*, p value cannot be assessed due to small number within strata.

Additionally, Gatalica and colleagues also documented a high rate of PD-L1 expression (45%) resulted from studying a cohort of 116 BC cases (Gatalica et al., 2014). On the other hand, Wimbery et al., (2015) and Chen et al., (2017) studied PD-L1 expression in advanced breast carcinomas, and they reported a high frequency of PD-L1

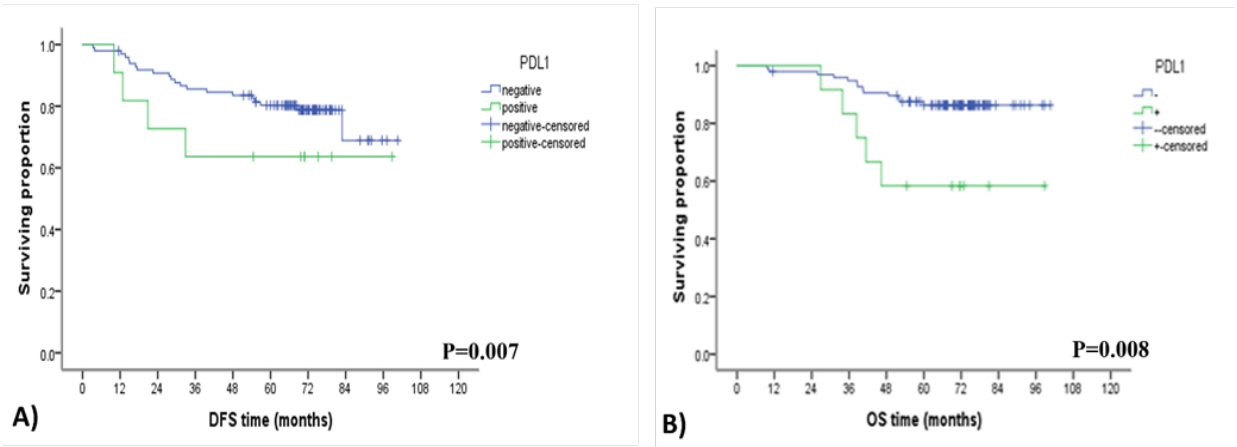

Figure 3. A) Association of PD-L1 Expression with Patients' Survival . A) Kaplan-Meier curves of disease-free survival between cases with positive and negative PD-L1 IHC expression. B) Kaplan-Meier curves of overall survival between cases with positive and negative PD-L1 IHC expression.

expression (49% and 30%, respectively). This is attributed to the fact that PD-L1 expression in tumor cells is strangely associated with aggressive biological behavior of the tumor and bad prognosis (Wu et al., 2019).

We observed in our study that PD-L1 expressed differently among different molecular subtypes. The highest PD-L1 expression was achieved in TNBC (16%), followed by Her2-enriched group (14%), while it was totally negative in Luminal A group. Unfortunately, this difference among subtypes was not statistically significant. This could be related to our limited sample size of each molecular subtype. Similar to us, Gatalica et al., (2014) and Kim et al., (2017) also found high PD-L1 expression in both TNBC and HER2 positive subtypes as compared to luminal subtypes.

On the contrary, Tsang and colleagues studied 1,091 BC patients. PD-L1 expression was higher in the luminal A subtype (34.1%) than that of the other BC subtypes (Tsang et al., 2017). We recommend further studies with larger samples of luminal A subtypes to make a conclusion about the usefulness of immunotherapy in this particular group of patients.

In the present study, the PD-L1 expression was more frequent in young age group (≤50 years), and large tumor size. There was also a strong association with high tumor grade and the development of distant metastasis. Tumor grade proved to be an independent prognostic factor for PD-L1 expression. The same results were reported by the studies of Kitano et al., (2017), Okabe et al., (2017) and Guo et al., (2020). It has been documented that PD-L1 expression is associated with poor prognostic factors, including high grade, large tumor size and positive l. node metastasis. However, there was a difference in our study, where PD-L1 showed a slight predominance in negative node cases.

We also found that PD-L1 expression is strongly associated with the type of tumor infiltrating lymphocytes (TILs). PD-L1 showed marked expression in tumors with FOXP3+ TILs and low CD8 infiltrate, while it did not reveal any expression in tumors with FOXP3- TILs and high CD8 infiltrate. This was in concordance with previous results Kitano et al., (2017), Okabe et al., (2017) and Guo et al., (2020). Our findings suggest that PD-L1 expression in early-stage BC is associated with immune response suppression.

Moreover, we showed that PD-L1 expression is significantly associated with worse OS and DFS of BC patients, independent of all studied prognostic factors. In a large metanalysis study (2,546 women) done by Zhang et al., (2017), they showed that PD-L1 overexpression was associated with worse prognosis and shorter overall survival. In another study, PD-L1 positivity was also associated with poor DFS, however there was no effect on OS (Kim et al., 2017). On the contrary, other reports showed better outcome in ER-negative or TNBC (Sabatier et al., 2015; Arias-Pulido et al., 2018; Humphries et al., 2018; Sobral-Leite et al., 2018).

Sobral-Leite et al., (2018) attributed these inconclusive results to the variability of the proportion of PD-L1 positivity in BC and the diversity of the assessment methods, using TMAs or small tumor material, as well as patient selection.

In conclusion, our study suggests that employing PD-L1 as a prognostic biomarker can help in stratifying early-stage BC patients and identifying those who are candidate for immunotherapy. Using 1% as a cut off, we showed that PD-L1 has a negative impact on patients' outcomes, especially those with high grade BC. Additionally, we showed that PD-L1 has a role in tumor immune response suppression in a subset of early-stage BC.

Author Contribution Statement

All authors contributed to this study. Preparation, data collection, review of the slides and analysis were performed by Dr Nancy H. Amin. Monitoring of data collection, interpretation of results, revision and guidance were done by Dr Saad Eissa, Dr Amany A. Abou-Bakr, Dr Hanan R Nassar and Dr Ghada Mohamed. The preliminary draft of the manuscript was written by Dr Nancy H. Amin and Dr. Ghada Mohamed. All authors revised and commented on primary version of the manuscript and approved the final one. Dr. Tamer S. Eissa helped in data analysis and revision of results.

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Ethical approval

The study was approved by the Institutional Review Board (IRB) no. IRB00004025 of National Cancer Institute (NCI), Cairo University. Oral and written informed consents were obtained from all patients or from their eligible relatives.

Availability of data

The datasets are available from the corresponding author on reasonable request.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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