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Programmed Cell Death Ligand 1(PD-L1) Expression in Molecular Subtypes of Breast Cancer

Fatma El-Zahraa Salah El-Deen Yassin[1]*, Cherry Gamal Gaballa Shehata[2], Eman Muhammad Salah EL-Deen[1]

[1]Department of Pathology, Faculty of Medicine, Sohag University, Sohag, Egypt

[2]Department of Pathology, Sohag Oncology Center, Sohag, Egypt

***Corresponding author:** Fatma El-Zahraa Salah El-Deen Yassin, **Mobile:** (+20)01007970144,

Email: fatmafm2002@yahoo.com

ABSTRACT

Background: Tumor infiltrating lymphocytes and programmed cell death ligand 1 (PD-L1) are un- classic prognostic indicators for breast cancer, they are major players in the immune response. Binding of Programmed cell death protein (PD-1) with PD-L1 plays an important role in the immune suppression and the process of tumor escape.

Objective: To evaluate the expression of PD-L 1 in mammary invasive duct carcinoma and its correlation with molecular subtypes and other histopathological parameters.

Patients and methods: Fifty paraffin blocks were included. Histopathological examination of H&E and PD-L1 immunostained sections were done. Scores of PD-L1expression: intra tumoral and in the tumor infiltrating lymphocytes (TILs) were evaluated. Molecular subtypes and other classic prognostic parameters were assessed (Tumor size, grade, stage, lympho vascular invasion (LVI) and *in situ* component).

Results: PD-L1 expression in TILs was more frequent than intra tumoral expression in various molecular subtypes. However, intra tumoral PD-L1 expression was associated with increasing tumor size, grade, the high level of TILs, LVI and molecular subtypes with special trend in Triple Negative Breast Cancer (TNBC) subtype. TNBC achieved the highest relative ratio of positive PD-L1 expression in both: intra tumoral and in TILs.

Conclusion: PD-L1 expression was more pronounced in TILs (immune cells) as compared with tumoral cells in all molecular subtypes of breast cancer with maximum ratio in TNBC. These results provide a promising future of PD-L1 as a target therapeutic option.

Keywords: TILs, PD-L1, TNBC.

INTRODUCTION

Breast cancer accounts for 25% of all malignancy and for 16.67 % of all cancer deaths, occupying the first rank for incidence and mortality in most of the countries [(1)].

Molecular alterations are known to affect cancer occurrence and metastasis, which has led to the development of hormonal therapy that targets the estrogen receptor (ER), progesterone receptor (PR), or human epidermal growth factor receptor type 2 (HER-2). However, up to 20% of patients with breast cancer experience disease progression and death, which highlights the need for more effective therapy [(2)].

Programmed cell death ligand 1 (PD-L1, also known as B7-H1) that was discovered in a variety of epithelial cancers is believed to mediate local immune evasion by binding to programmed cell death 1 (PD-1), its co-stimulatory receptor on T cells, to induce saturation of activated anti-tumor T cells [(3)].

PD-1 and PD-L1 have been shown to be promising targets for the treatment of different tumor types. Anti-PD-1 therapies were effective for breast cancer, and particularly triple negative breast cancer (TNBC) [(4)].

Aim of the work was to evaluate the expression of PD-L1 in invasive duct carcinoma of the breast and its correlation with molecular subtypes and other histopathological parameters.

Patients and specimens:

Fifty formalin-fixed paraffin embedded tissue blocks of 50 cases of mammary duct carcinoma were collected from the archives of the Pathology Department, Sohag University Hospital and Sohag Oncology Center during the period (January-December 2020). Tumor samples included total, subtotal mastectomy and excisional specimens.

Ethical Approval:

Ethical approval was obtained from the Institutional Research Ethical Committee. Data about patients' age, tumor size, ER, PR, HER-2/neu and Ki-67 status were obtained from clinical reports.

Histopathological evaluation:

Four micrometer (4µm)-thick tissue sections were prepared from the tissue blocks and stained with hematoxylin and eosin stains. The tumors were reviewed for tumor histological type according to the WHO classification [(5)], graded and staged pathologically (p) according to WHO recommendations [(5)].

Tumor infiltrating lymphocytes (TILs) were assessed according to the international TIL Working Group Consensus guidelines 2014[(6)]. They were

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evaluated on H&E stained section and scored into low and high according to the predefined criteria (more than 50% is lymphocytic predominant).

Ki67 was considered high and low with cutoff point 14% [7]. Tumors were grouped according to the molecular subtypes: luminal-A like, luminal-B like, HER-2 positive and TNBC[8].

Immunohistochemical staining:

Four micrometer (4µm)-thick sections were prepared on positively charged slides, de-paraffinized in xylene for 20 minutes (min), rehydrated in downgraded alcohol (100%, 80%, 70% and 50%) 2 min for each and rinsed in distilled water.

Tissue sections were incubated in hydrogen peroxide (10%) for 10 min to block endogenous peroxidase activity followed by washing twice in phosphate buffer saline (PBS). A Pascal Dako pressure cooker was used for antigen retrieval. The slide was immersed in EDTA (pH 8.9) for 30 minutes, dried at room temperature, and washed in distilled water. Tissue sections were incubated overnight in a moist chamber, with Rabbit Anti-PD-L1 (CD274: RM0324, RM0324RTU7) Medaysisat monoclonal antibody (1/100) at 4ºC.

The slides then washed in phosphate-buffered saline (PBS), pH 7.4 to 7.6. As detection system, the slide was incubated in ADVANCE HRP Detection System (Dako, Carpinteria, CA) at 37°C for 1 hour and washed in PBS. After that, 3,3′-diaminobenzidine chromogenic substrate was applied at the proportion 0.06 g to 100 mL of PBS, 500-µL 3% hydrogen peroxide, and 1-mL dimethylsulfoxide at 37°C for 5 minutes.

Sections were counterstained by immersion in hematoxalin stain for a few seconds and rapid wash in tap water to remove extra dye. Dehydration, clearance, and cover mounting were done. Sections from tonsils were used as positive control for PD-L1. Also negative controls were lacking reactivity to confirm the validity of the staining results.

Immunohistochemical evaluation and scoring of PD-L1:

The scoring was done for both tumor cells and tumor infiltrating lymphocytes (TILs). Partial or complete membranous staining of tumor cells is considered and included within the score. The expression of tumor cells was evaluated by percentage and modified H score (MHS). MHS ranges from 0-300 by detection of both staining intensity and percentage of stained tumor cells then summation of individual H score of each intensity. The intensity of staining ranges from 0:3 (0=negative, 1=low, 2=moderate, 3=strong); (1x % cells of score 1 + 2x % cells of score 2 + 3x % cells of score 3). Using cut off score ≥ 100 (0-99= negative -low expression, 100-300=high expression)[9].

The expression of tumor infiltrating lymphocyte was evaluated by the percentage; both membranous and/or cytoplasmic staining of immune stromal cells (lymphocytes and macrophages) are considered with cut off point > 1% [10].

Statistical Analysis

Data were statistically analyzed using IBM SPSS Statistics for Windows version18. Quantitative data were expressed as means ± standard deviation, median and range. Qualitative data were expressed as number and percentage. The data were tested for normality using the Shapiro-Wilk test. Chi-Square test was used to evaluate statistical significance of various parameters with p value less than <0.05 was considered statistically significant.

RESULTS

The mean age of the studied patients' ±SD was 51.8 ± 13.4 years. The mean tumor size was 3.8 ± 2.97 cm. ER expression and PR expression were positive in majority of cases 70% and 68%; respectively, while HER2/neu expression was positive in 22% of our studied cases. Ki-67 expression level was high in 54% of the studied cases.

The H&E stained sections of the 50 studied cases were reviewed and diagnosed invasive duct carcinoma (IDC) with ductal carcinoma insitu (DCIS) component in 50% of the studied cases. They were graded according to the WHO grading criteria into: Grade I; 5/50 (10%), Grade II; 34/50 (68%) and Grade III; 11/50 (22%) tumor. TILs of the studied cases had low level in 24/50 (48%) and high level in 26/50 (52%) (Table 1).

Lympho-vascular invasion (LVI) was found in 17/50 (34%) of the studied cases. Regional lymph node metastasis was positive in 26/50 (52%) of the studied cases. Regarding tumor size (T); 9/50 (18%) of the studied cases were T1, 34/50 (68%) were T2, 3/50 (6%) were T3 and 4/50 (8%) were T4. Concerning lymph node status (N); 24/50 (48%) of the studied cases were N0, 7/50 (14%) were N1, 12/50 (24%) were N2 and 7/50 (14%) were N3.

Regarding molecular subtypes; luminal A- like subtype was 42%, luminal B- like subtype was 30%, HER2 positive subtype was 12% and TNBC was 16% of the studied cases (Table 1). We obtained the ER, PR, HER2 and Ki-67 stained slides from the archive.

Table (1): The correlation between Tumoral PD-L1 expression and the histopathological parameters.

Parameters		Total N=50	Tumoral PDL-1 expression (cutoff point ≥100)		P-value
			Positive PD-L1 (100-300)	Negative/low PD-L1 (0-99)	
Tumor size (cm)					
≤ 2 cm		**14**	**0 (0%)**	**14 (100%)**	**0.039**
>2 cm		**36**	**9 (25%)**	**27 (75%)**	
Grade	**I**	**5 (10%)**	**0 (0%)**	**5 (100%)**	**0.001**
	II	**34 (68%)**	**1 (2.9%)**	**33 (97.1%)**	
	III	**11 (22%)**	**8 (72.7%)**	**3 (27.2%)**	
DCIS		**25 (50%)**	**6 (24%)**	**19 (76%)**	**0.269**
TILs	**Low**	**24 (48%)**	**0 (0%)**	**24 (100%)**	**0.001**
	High	**26 (52%)**	**9 (34.6%)**	**17 (65.4%)**	
Molecular subtypes	**Luminal A-like**	**21 (42%)**	**1 (4.8%)**	**20 (95.2%)**	**0.044**
	Luminal B-like	**15 (30%)**	**3 (20%)**	**12 (80%)**	
	HER2-positive	**6 (12%)**	**1 (16.7%)**	**5 (83.3)**	
	TNBC	**8 (16%)**	**4 (50%)**	**4 (50%)**	
TNBC		**8**	**4 (50%)**	**4 (50%)**	**0.001**

Chi-square test was used, p-value less than 0.05 was considered statistically significant.

PD-L1 expression in tumor cells:

Brownish membranous staining of tumoral cells with PD-L1 was reported as positive expression **(Figure 1)**. Using H-score, cutoff point of ≥100, PD-L1 expression was positive in 9/50 (18%) of the studied cases **(Figure 2)** and showed significant association with tumor size (p=0.039), tumor grade (p< 0.001), TILs (p=0.001), molecular subtypes (p=0.044) and TNBC subtype (p= 0.01). However, statistical evaluation of PD-L1 expression in relation to T or N stage, presence of LVI or DCIS component **(Figure 3)**, ER, PR, HER-2 status and Ki-67 index showed no statistical significance.

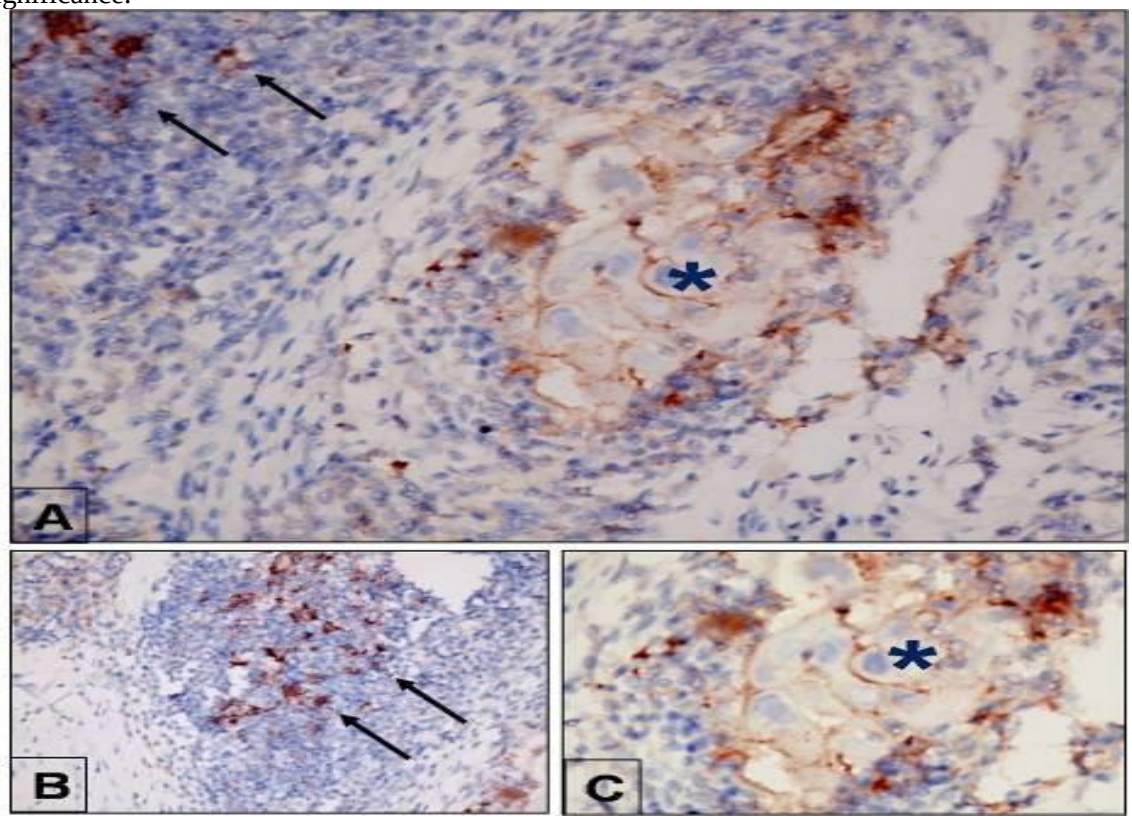

Figure (1): Double expression of PD-L1 in mammary invasive duct carcinoma (IDC) with high TILs. A&C) Membranous brown staining of tumoral cells (dark blue astrix). B) Punctuate brown cytoplasmic staining of TILs (arrows)(PD-L1 x400,200,400. Original).

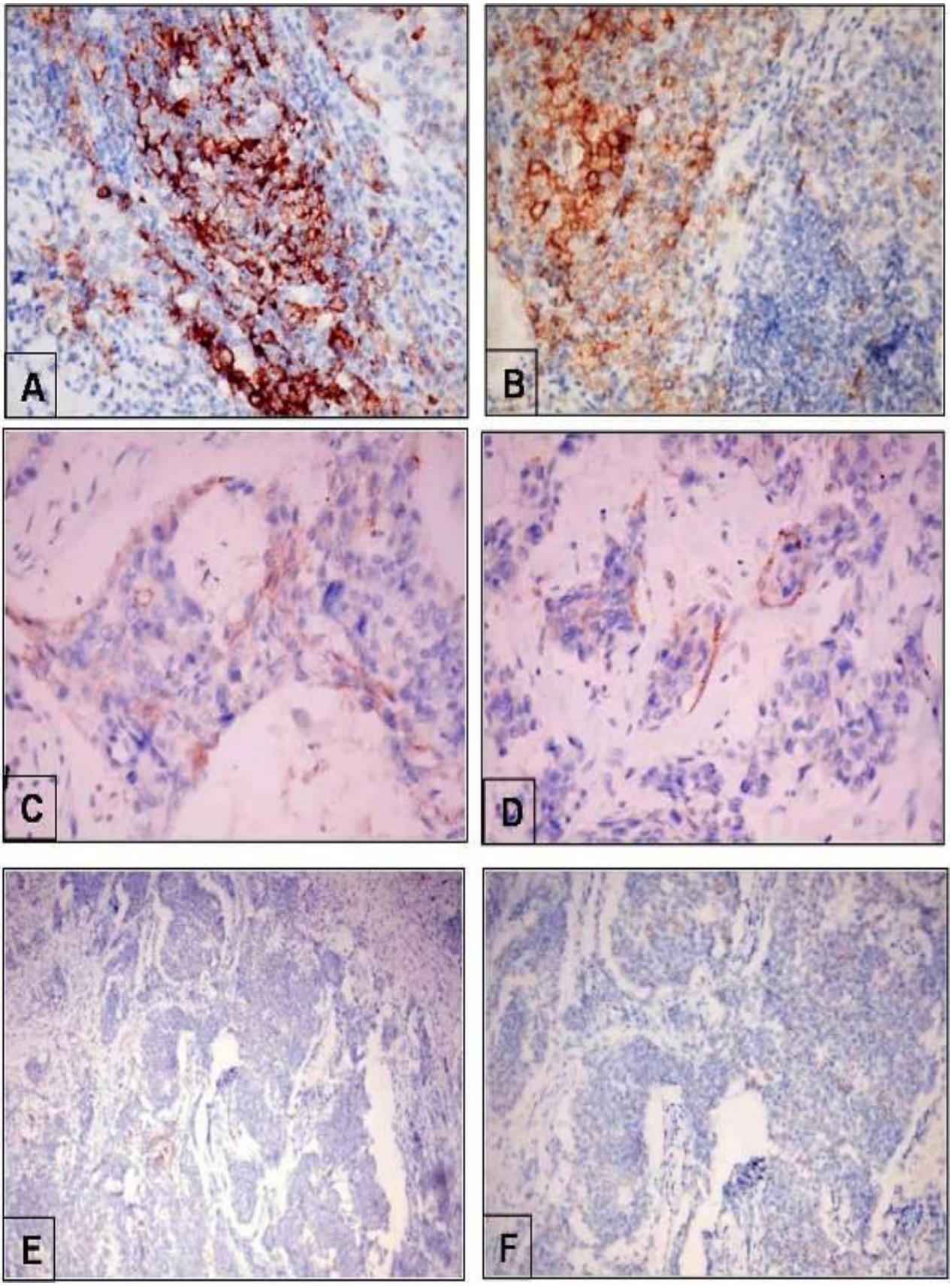

Figure (2): Variable scores of PD-L1 immunostaining in IDC. A-B) High Score of PD-L1 with intra tumoral expression. C-D) Low Score of PD-L1 with intra tumoral expression. E-F) Negative intra tumoral expression of PD-L1. (PD-L1 400,400,400,400,100,200. Original).

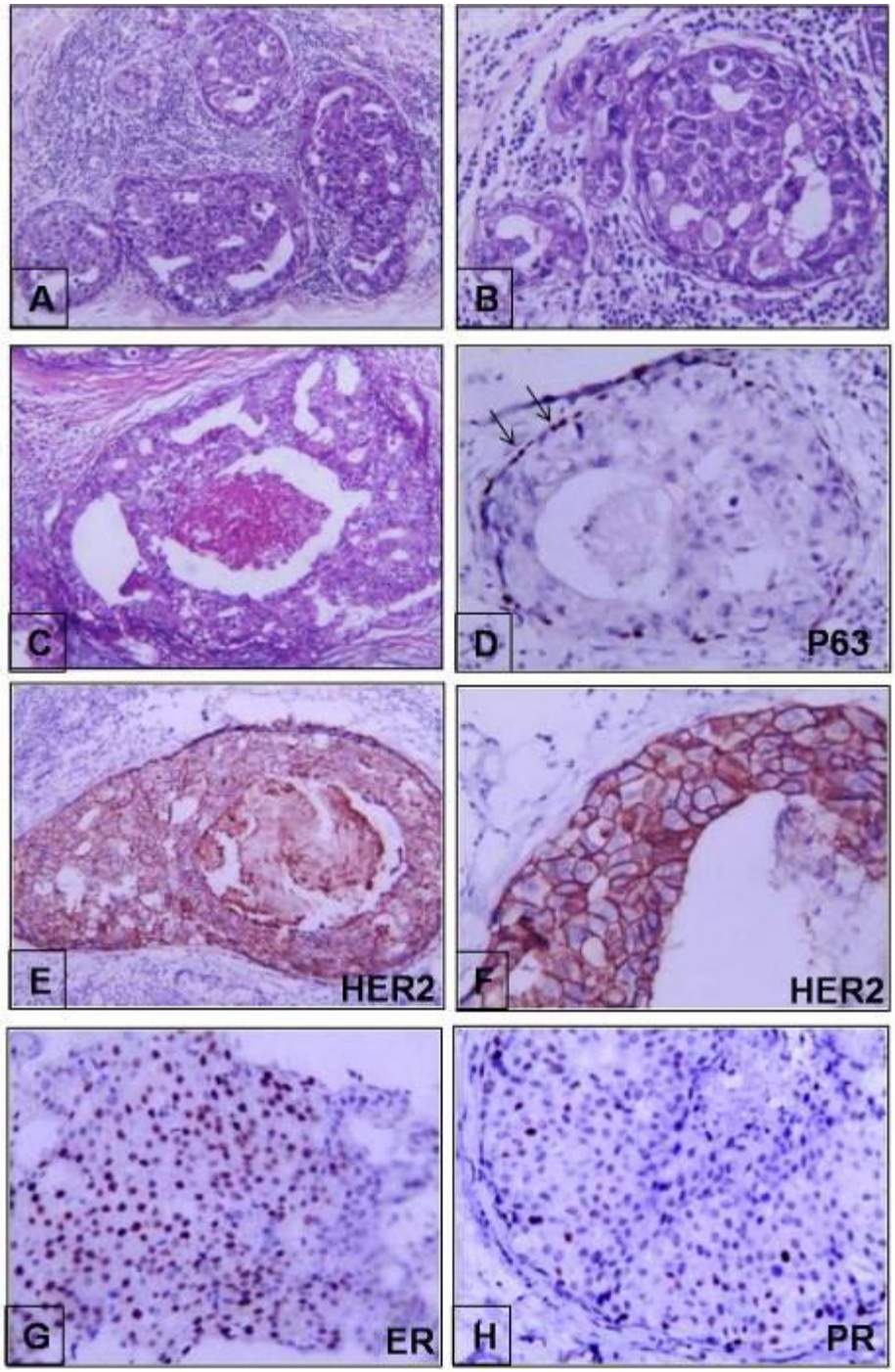

Figure (3): A case of ductal carcinoma insitu with dense lymphocytic infiltrate . A-C) Variable foci of DCIS (cribriform and comedo patterns) (HEx40,100,100 original). D) P63 confirm the diagnosis and highlight the myoepithelial layer (arrows) (x100). E-F) strong membranous staining of HER2 (x100,400). G&H) Positive nuclear staining of ER and PR. PD-L1 was negative (image not included)(x100,100. Original).

PD-L1 expression in TILs:

PD-L1 protein expression in TILs appeared as punctuate brownish staining. PD-L1 protein expression in TILs was positive in 23/50 cases (46%). PD-L1 expression in TILs was significantly associated with tumor grade ($p=0.001$) and TILs ($p<0.001$) (Table 2). However, statistical evaluation of PD-L1 expression in relation to T stage, N stage, LVI, presence of DCIS component, ER, PR, HER2 status, Ki-67 index, and molecular subtypes showed no statistical significance.

Table (2): The correlation between PD-L1 expression in TILs and the histopathological parameters.

Parameters		Total N=50	Immune cell expression of PD-L1 (cutoff point ≥1%)		p-value
			Positive PD-L1	Negative PD-L1	
Grade	**I**	**5 (10%)**	**0 (0%)**	**5 (100%)**	**0.001**
	II	**34 (68%)**	**13 (38.2%)**	**21 (61.8%)**	
	III	**11 (22%)**	**10 (90.9%)**	**1 (9.1)**	
DCIS		**25 (50%)**	**17 (68%)**	**8 (32%)**	**0.382**
TILs	**low**	**24 (48%)**	**0 (0%)**	**24 (100%)**	**0.001**
	High	**26 (52%)**	**23 (88.5%)**	**3 (11.5%)**	
Molecular subtypes	**Luminal A-like**	**21 (42%)**	**6 (28.6%)**	**15 (71.4%)**	**0.097**
	Luminal B-like	**15 (30%)**	**7 (46.7%)**	**8 (53.3%)**	
	HER2-positive	**6 (12%)**	**4 (66.7%)**	**2 (33.3)**	
	TNBC	**8 (16%)**	**6 (75%)**	**2 (25%)**	
TNBC		**8**	**6 (75%)**	**2 (25%)**	**0.073**

P- Value was calculated by Chi-square test, P-value less than 0.05 is statistically significant

We estimated the relative ratio between the number of cases with intra tumoral positive expression of PD-L1 **TO** the number of cases with positive PD-L1 expression in tumor infiltrating lymphocytes (Table 3) in all molecular subtypes **(Figures 4 & 5)**.

Table (3): The ratio between the number of positive cases with intra tumoral PD-L1 expression and the number of cases with positive PD-L1 expression in the TILs

PD-L1 expression		No of cases =50	Luminal A-like N=21	Luminal B- like N=15	HER2-positive N= 6	TNBC N=8
PD-L1 expression in tumor cells (Cut off point ≥100)	Negative/weak	41 (82%)	20	12	5	4
	Positive	**9 (18%)**	**1**	**3**	**1**	**4**
PD-L1 expression in TILs (Cut off point >1%)	Negative	27 (54%)	15	8	2	2
	Positive	**23 (46%)**	**6**	**7**	**4**	**6**
Ratio=No of positive cases with intra tumoral PD-L1 expression / No of positive cases with TILs PD-L1 expression			**1:6 (0.16)**	**3:7 (0.43)**	**1:4 (0.25)**	**4:6 (0.67)**

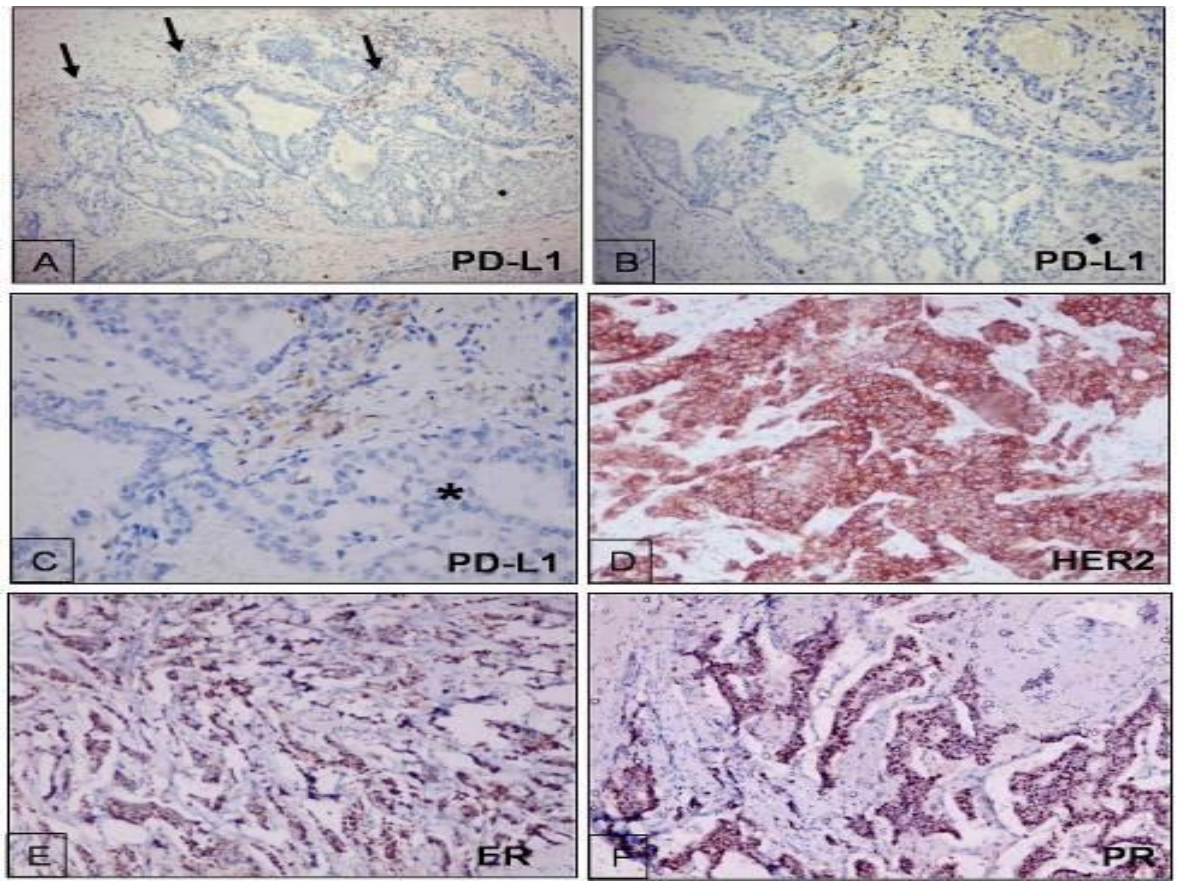

Figure (4): Luminal B-like with HER2 positive case of IDC. A-C) Negative expression of PD-L1 in the tumor cells (astrix) while positive expression in TILs at the periphery (black arrows) (X100,200,400. Original) . D-E) Positive HER2 (membranous), ER (nuclear) and PR (nuclear) (all x400. Original).

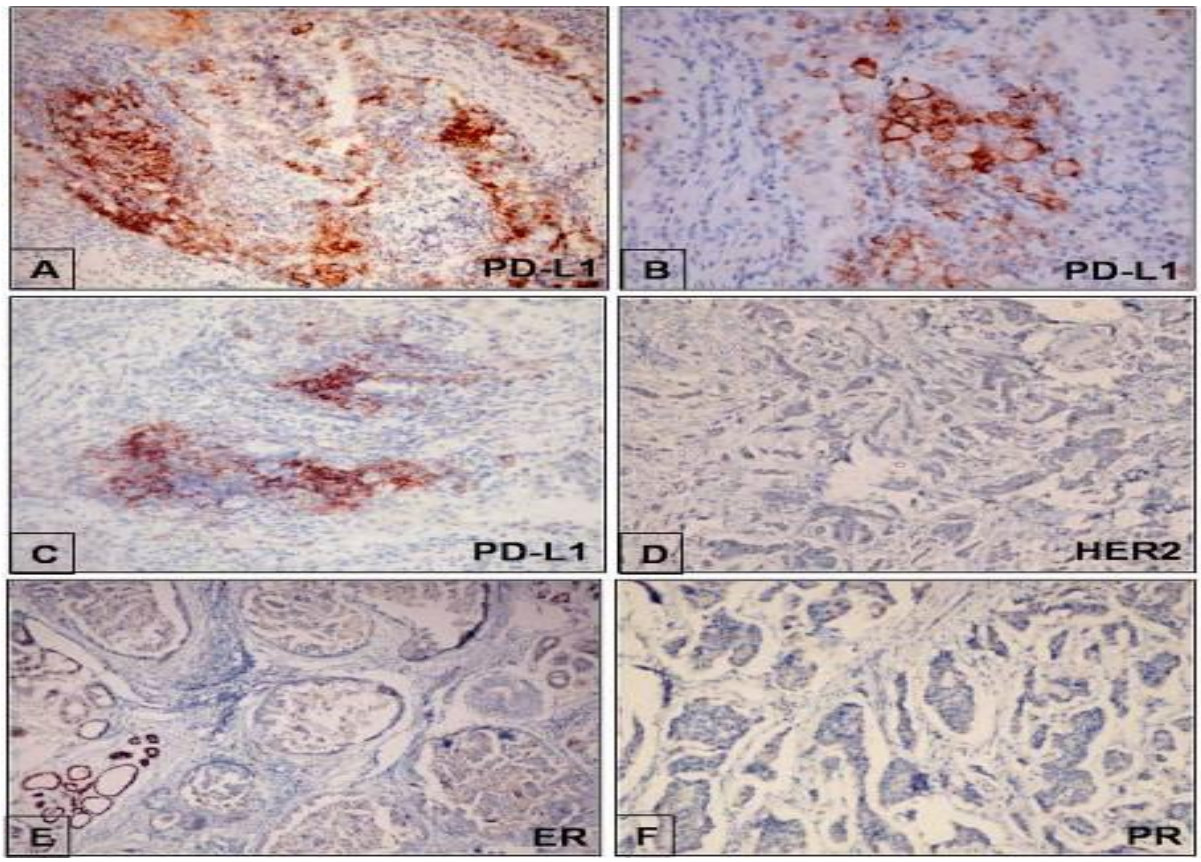

Figure (5): Strong expression of PD-L1 in TNBC. A) Positive immuno-staining of both tumoral cells and TILs (immune cells). B) Strong membranous staining of tumoral cells. C) Punctuate brownish staining of TILs. D-F) Negative expression of HER2, ER (with internal positive control) and PR respectively. (PD-L1 x200, 400,200-X200,200,400. Original).

DISCUSSION

Several prognostic factors control the management of breast cancer. The classic factors such as: tumor size, tumor grade, stage and lympho vascular invasion. Other factors like tumor infiltrating lymphocytes and PD-L1 expression were reported as promising prognostic indicators of breast cancer.

Firstly, we will assess the classic factors: Tumor size is a strong independent prognostic factor for patient survival and prognosis. Women with invasive tumors measuring >2cm have higher risk of lymph node metastasis [(11)] and we used the mentioned size to categorize our patients**.**

In the current study, the regional lymph node metastasis was negative in 48% of cases, which close to what was reported by **Zhou *et al.*** [(12)] who found that 46% of their studied cases showed negative regional lymph node metastasis.

Regarding tumor grade; the current study included 10% grade I, 68% grade II and 22% grade III tumor. These results were near to those recorded by **Elkhodary *et al.*** [(13)] who found 7.2% of cases were Grade I, 73.8% of cases were Grade II and 19% of cases were Grade III.

Tumor grade has the same prognostic value of lymph node status and greater than that of tumor size in predicting the prognosis and outcome in patients, also it is useful in selecting neo-adjuvant chemotherapy [(12)].

Lympho-vascular invasion (LVI) is an independent and poor prognostic factor in cases of invasive breast cancer; the analysis of LVI may have great prognostic significance particularly in luminal A like patients with subsequently greater relevance in the decision for adjuvant therapy[(14)]. LVI was observed in 34% of cases in the current study in agreement with **Öz *et al.*** [(15)] who reported LVI in 31% of their cases.

Regarding molecular subtypes; luminal A and luminal B like subtypes represented 36/50(72%) of the studied cases in the present study, while, HER2 positive subtype represented 6/50 (12%) and TNBC represented 8/50 (16%) which was in agreement with **Abdelshafy *et al.*** [(16)] who found that luminal A and luminal B like subtypes were 73%, HER2 positive subtype was 10.2% of cases and TNBC was 16.6% of the studied breast cancer cases.

Several authors [(6 &7& 12)] reported TILs an established prognostic biomarker in breast cancer. Increased TILs levels eliminate the tumor cells and can predict a better response rate to immunotherapy [(17)]. Both TILs and PD-L1 expression are immune responses in breast cancer.

The programmed cell death ligand (PD-L1) is an immune check point protein expressed in tumoral and stromal immune cells. Binding of PD-L1 with its protein (PD-1) leads to apoptosis of immune cells especially T cells followed by more tumor progression. Block of the previous reaction is controlled by immune checkpoint inhibitors such as PD-L1 inhibitors [(18)].

In the current work we assessed the expression of PD-L1 in different molecular subtypes of the breast cancer and its correlation with other histopathological parameters.

We found a statistically significant association between PD-L1 expression and high level of TILs ($p<0.001$), in agreement with **Chen *et al.*** [(19)] who found also a high significant association between PD-L1 expression and high TILs with similar P values (0.001).

TNBC achieved the highest score of PD-L1 expression intra tumoral and in the lymphocytic infiltrating tumor cells in the current study, the luminal types recorded the lowest H score and in lymphocytic infiltrating tumors cells.

Furthermore, we detected a strong positive association between intra tumoral PD-L1 expression and PD-L1expression in TILs ($p <0.001$). This finding was in agreement with **Kim *et al.*** [(20)] who found a strong association between PD-L1 expression in tumor cells and PD-L1expression in TILs.

In the current study, a cutoff point of ≥1% was used to evaluate PD-L1protein expression in TILs; which is the reported threshold for clinical response to PD-L1 inhibitors in non-small-cell lung carcinoma and has also been reported in breast carcinoma [(10)].

An interesting relative Ratio could be highlighted in the current work when we evaluated the number of positive cases with intra tumoral PD-L1 expression in relation to the number of cases with positive PD-L1 expression in the TILs in every molecular subtype of breast cancer.

Patients with triple negative breast cancer (TNBC) showed negative expression of ER, PR and HER2. They represented 16% of our cases with similar percentage to others. TNBC reported the highest ratio (2:3) (No of positive cases with intra tumoral PD-L1 expression: No of positive cases with TILs PD-L1 expression). We mean that two cases out of three of TNBC showed double sites PD-L1 expression: intra tumoral and in TILs.

While the other molecular subtypes revealed variable lower ratio of PD-L1 expressions: Luminal A-like (1:6), Luminal B-like (3:7) and HER2 positive (1:4).

There are conflicting results as regard PD-L1 in TNBC, the majority showed improved outcome with increase PD-L1 level while some recorded the opposite **Zhang and his collegues** [(21)]. Additionally, there are many scoring systems with variable Cutoff points for assessment of PD-L1 expression and most of them are site dependant. Thus we used the previous relative ratio to assess the expression of PD-L1 in all molecular subtypes of breast cancer whatever the used score.

Increased PD-L1 positive TILs improve response to the neo adjuvant chemotherapy in all molecular subtypes of breast cancer and is associated with longer survival in HER2-positive and TNBC [(22)].

Significant positive association between PDL-1 expression and tumor grade ($p<0.001$), a finding similar to what was reported by **Kitano *et al.* [23]** who found positive association between PDL-1 expression and tumor grade.

PD-L1 expression in TILs showed significant association with tumor grade ($p<0.001$); positive PD-L1 expression in TILs was detected in 90.9 % of grade III cases. **Kim *et al.* [20]** found that 82.4 % of grade III cases have high positive PD-L1 expression in TILs.

Our study revealed highly significant association between PD-L1 expression in TILs and high level of TILs ($p<0.001$) as positive expression was observed in 76.9 % of cases with high level of TILs. This was similar to the findings of **AiErken and his colleagues [17]** who observed positive expression of TILs in 77% of cases with high level of TILs.

We detected 50% of IDC with in situ components which showed no PD-L1 expression in comparison to the adjacent invasive areas. **Huang *et al.*[24]** mentioned large tumor size, high tumor grade, negative ER and/or PR and TNBC subtype are unfavorable prognostic factors which were associated with PD-L1 positive tumor cells.

The classic treatment options for breast cancer include anti-Her2 for Her-2 enriched subtype and hormonal therapy for luminal subtypes. There is no approved targeted treatment available for metastatic TNBC patients. Immune-therapy comprises a promising new era in breast cancer therapy. One of the most extensively used active immune-therapy is immune checkpoint blocking[25].

Doğukan *et al.*[26] reported that increased PD-L1 expression and an up-regulated level of TILs in TNBC provided an ample opportunity for the immunotherapy to eliminate tumor cells.

We concluded that PD-L1 expression was more pronounced in TILs (immune cells) as compared with tumoral cells in all molecular subtypes of breast cancer with maximum ratio in TNBC, this creates a chance for more promising future of PD-L1 as a target therapeutic option.

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