



Prognostic implication of PD-L1 expression and associated tumor infiltrating lymphocytes in metastatic breast cancer

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Abstract

Introduction: One of the most important regulators of immune response is the programmed death receptor 1 (PD-1) and its interaction with its ligand (PD-L1), which negatively influences the immune response.

Objectives: This study aims to clarify PD-L1 expression levels and the associated tumor infiltrating lymphocytes (TILs) in patients with metastatic breast cancer, and to assess their influence on the prognosis of these patients and the association with clinico-pathologic criteria.

Patients and Methods: PD-L1 expression was analyzed using immunohistochemistry (IHC) while TILs count was assessed by histopathological examination of the hematoxylin and eosin (H&E) stained full tumor sections from 50 patients diagnosed with stage IV breast cancer at Ain Shams University hospital, Cairo, Egypt.

Results: PD-L1 expression was demonstrated on TILs in 21 of 50 specimens, and on tumor cells in 13 of 50 specimens. Triple negative breast cancer (TNBC) and ER-/Her2+ subtypes were significantly associated with TIL infiltration and PD-L1 expression (on TILs and tumor cells). High TIL infiltration was significantly associated with worse overall survival (OS) and progression free survival (PFS) ($P=0.0238$ [HR 4.7, 95% CI: 1.22-18.11] and $P=0.0262$ [HR 3.1, 95% CI: 1.14-8.59] respectively). No correlation was found between PD-L1 expression (on tumor or TILs) and the survival of the patients (OS nor PFS).

Conclusion: High TIL count infiltrating the breast tumor is associated with worse OS and PFS in patients with metastatic breast cancer. High PD-L1 expression correlated with high counts of TIL levels around the tumor. These findings have major clinical implications in using immune-checkpoint inhibitors in treating breast cancer patients.

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Introduction

Globally, breast cancer is the second most frequently diagnosed malignancy just behind lung cancer, accounting for over two million cases each year, and the fourth cancer causing death worldwide; the leading cause of cancer death though in females, with around 600 000 deaths each year (1).

Cancer immunotherapy through inhibition of negative immune regulators or checkpoints, represents a major progress in cancer therapy. Of the immune checkpoints to be targeted is the programmed cell death protein 1 (PD-1) and its ligand (PD-L1). The measurement of these two biomarkers is used as a prognostic and predictive marker to the immunotherapy with checkpoint inhibitors (2).

Inhibitors of PD-1 and PD-L1 were shown to significantly improve patient outcomes in different types of immunogenic cancers such as non-small cell lung cancers, malignant

Key point

The role of PDL1 expression in breast cancer is still under investigation for possible application of immunotherapy as a new treatment modality. Triple negative breast cancer (TNBC) and ER-/Her2+ subtypes were significantly associated with tumor infiltrating lymphocytes (TILs) infiltration and PD-L1 expression (on TILs and tumor cells). High TIL infiltration was significantly associated with worse overall survival (OS) and progression free survival (PFS). High TIL counts and PD-L1 positivity (on both TILs and tumor cells) were most significantly associated with ER-/Her2+ and TNBC. Further studies with large sample size are recommended to find correlation between PD-L1 expression (on tumor or TILs) and the survival of the patients (OS nor PFS).

melanoma, renal cell carcinoma, and some immunogenic breast cancer subtypes (3-6).

Breast cancer is composed of heterogeneous subtypes; each has its specific pathologic and genetic characters, as well as different

prognostic significance. Of these subtypes, triple negative breast cancer (TNBC) is the most immunogenic type, as it is rich in tumor infiltrating lymphocytes (TILs) and PD-L1 co-expression (7).

In a phase III trial, combining atezolizumab and nab-paclitaxel conferred a non-statistically significant overall survival (OS) benefit compared with nab-paclitaxel alone in unselected, patients with TNBC. Interestingly, the difference in OS was statistically and clinically significant in the PD-L1 positive subgroup of patients, highlighting the potential clinical importance of PD-L1 expression as a predictive biomarker in breast cancer (8,9).

Objectives

We aimed to evaluate the prognostic value of PD-L1 expression and associated TILs in stage IV breast cancer patients, and correlate them with response evaluation, progression free survival (PFS) and OS.

Patients and Methods

Patients

Tumor samples were collected at Demerdash Pathology Lab from 50 female patients with pathologically proven stage IV breast cancer between 2017 and 2019 presented at the breast cancer unit in Department of Clinical Oncology, Ain Shams University hospital, Cairo, Egypt. The ruled in patients did not receive any treatment yet to their metastatic disease. Patients who received adjuvant or neoadjuvant systemic treatment were enrolled in the study. Patients were followed up to at least one year after starting their systemic treatment. Patients with associated systemic immunologic diseases and pregnant patients were excluded from the study.

Scoring methods

The percentage of stromal TILs is determined by the area occupied by mononuclear inflammatory cells over total intra-tumoral stromal area within the borders of the invasive tumor (including the invasive tumor front).

Immunohistochemistry (IHC) staining was conducted on the paraffin embedded tissue sections with a labelled streptavidin- biotin-peroxidase complex technique using a rabbit monoclonal antibody for PD-L1 (CD274 molecule) (catalogue number: cell signaling, 13684; dilution: 1/200). Antigens were retrieved by microwaving in citrate buffer for 20 minutes for PDL1. To verify antibody specificity; placental tissue was used as a positive control for PDL1.

Scoring of TIL was based on the recommended methodology of the International TILs Working Group (10). The density of stromal TILs is recorded as a continuous percentage and categorized for analyses as; none or focal TILs if <5%, low in between 5–50%, and high if >50% (10).

PD-L1 scoring was conducted according to a previous study by Sobral-Leite et al. Cells with membranous

expression of PDL1 were considered positive, while positive cytoplasmic or nuclear staining were nonspecific. PD-L1-positive tumor cells (PD-L1+ tumor-cells) exhibiting membrane staining over the entire tumor were recorded as a continuous variable (0-100%) and categorized for analyses as: *negative* if <1% and *positive* if ≥1%. PD-L1-positive immune cells scoring was based on the percentage of the area occupied by stromal TILs exhibiting PD-L1 staining over the total area occupied by stromal TILs (0-90%; as continuous variable). It was categorized as; negative or focal if <5%, moderate if between 5-50%, and high if >50% (7).

Statistical analysis

Baseline characteristics of the patients and tumor pathological features are expressed as absolute values, mean ± standard deviation and median (range) when appropriate. A chi-square test was used to measure the association between these parameters. Survival data were presented on Kaplan-Meier curves and suitable tests were used to compare survival data. Results will be shown as *P* values. Statistical significance is defined as *P* < 0.05. Value of *P* < 0.01 is highly significant whereas the *P* > 0.05 is non-significant.

Results

The clinical and pathologic criteria of the patients enrolled in our study is summarized in [Table 1](#).

This study included 50 female patients with treatment naïve metastatic breast cancer, with mean age 45.8 years (standard deviation 11.1, range 25 to 75 years). From this analysis, 35 patients (70%) were pre-menopausal, while 15 (30%) were post-menopausal. Regarding the metastatic deposits, 14 patients (28%) had only bone metastasis, while 20 patients (40%) had only visceral deposits. 16 patients (32%) had *de novo* metastatic disease (metastatic from the start), while 34 patients (68%) were relapsed after adjuvant/neoadjuvant therapy. As regards the type of first line metastatic systemic therapy received, 29 patients (61.7%) received chemotherapy, 13 patients (27.7%) received endocrine therapy, while only five patients (10.6%) received combined chemotherapy plus endocrine therapy. Among the 50 patients of the study, 35 patients (70%) were of the luminal subtypes, 8 patients (16%) were ER negative/Her2 positive, while 7 patients (14%) were TNBC.

We searched for correlations between PD-L1 expression and TIL infiltration in tumor specimens for patients with metastatic breast cancer, and their relation to the clinico-pathological features of the patients.

We observed a strong correlation between TILs and PD-L1 expression (on tumor and surrounding TILs [*P* = 0.0011 and *P* < 0.0001 respectively; [Table 2](#)]).

TNBC and ER-/Her2+ subtypes were the most common subtypes significantly associated with presence of TIL

Table 1. The clinicopathologic data of the patients

Characteristic	Number (%)
Age (years)	45.8 ± 11.1
Menopausal status	
Post	15 (30)
Pre	35 (70)
Co-morbidities	
No	31 (62)
Yes	19 (38)
Tumor grade	
2	33 (66)
3	11 (22)
Missing	6 (12)
Tumor intrinsic subtypes	
Her2 enriched	8 (16)
luminal A	17 (34)
luminal B	12 (24)
luminal B Her2 enriched	6 (12)
TNBC	7 (14)
De novo metastatic disease	
No	34 (68)
Yes	16 (32)
Site of metastases	
Viscera only	18 (36)
Bone only	15 (30)
Viscera and bone	17 (34)
Burden of disease	
Multiple	45 (90)
Oligo	5 (10)
Type of first line systemic treatment	
CTH	29 (62)
CTH/ Endocrine	5 (10)
Endocrine	13 (28)

and positive expression of PD-L1 (Figures 1 and 2). We found that 85.7% of TNBC specimens and 100% of ER-/Her2+ subtypes were associated with TIL infiltration and positivity of PD-L1.

Regarding the survival data, patients with high TIL counts showed worse OS and PFS ($P=0.0238$ [HR 4.7, 95% CI 1.22-18.11] and $P=0.0262$ [HR 3.1, 95% CI 1.14-8.59] respectively) (Figures 3 and 4; Table 3).

Of note, there was no correlation between PD-L1 positivity (on tumor or immune cells) and survival of patients (OS and PFS) (Table 3).

Discussion

In breast cancer, the reported frequency of PD-L1

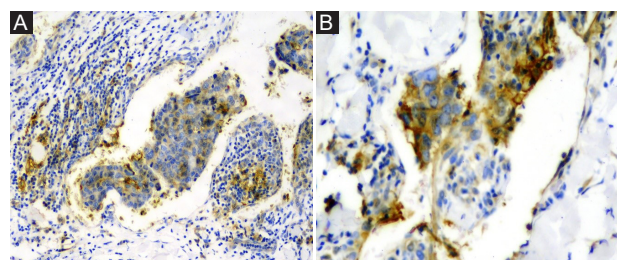


Figure 1. (A) Case of Invasive duct carcinoma grade 3 ER-/Her2 + showing moderate TIL PDL-1 expression (5-50%) and positive PDL-1 expression in tumor cells (>1%). (PDL1×200). (B) Same case showing strong positive membranous expression of PDL-1 in tumor cells surrounded by TIL (PDL-1 ×400).

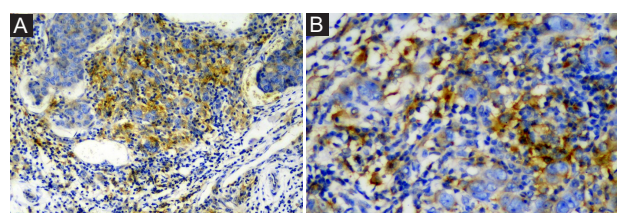


Figure 2. (A) A case of triple negative breast carcinoma grade 3 showing moderate TIL PDL-1 expression (5-50%) and positive PDL-1 expression in tumor cells (>1%). (PDL1×200). (B) Same case showing strong PDL-1 expression in 50% of TIL (PDL-1 ×400).

expression by tumor cells varies between studies (11-15). In a study that analyzed tissue from 44 patients with breast cancer, PD-L1 was expressed in 34% of the tumors using the mouse monoclonal MIH1 clone (13). In a subsequent study conducted by the same group and using an expanded version of the cohort ($n=69$), PD-L1 expression was expressed in 29% of breast carcinomas (14). In another report, PD-L1 was expressed in 23.4% of 650 breast cancer patients (11). A study analyzing tumors from 192 patients observed PD-L1 expression in 56% of breast cancers (16). Another study found that PD-L1 was expressed in more than 1% of tumor cells in only 1.7% of total 3916 breast tumors by IHC analysis (12). A study analyzing tissue from 636 patients using in situ mRNA hybridization revealed that PD-L1 was expressed in 58% of the breast cancer specimens in TMAs (17). Another study using mRNA expression and DNA microarrays found that PD-L1 gene expression was upregulated in 20% of all samples and 38% of basal tumors (15). All these differences may be explained by the absence of reliable antibodies, validated

Table 2. Correlation between TIL and PD-L1 on tumor cells and TILs

TIL	PD-L1 on TILs, No (%)			P value	PD-L1 on tumor cells, No (%)		P value
	Focal (n=29)	Low (n=12)	High (n=9)		Negative (n=37)	Positive (n=13)	
Focal	26 (100)	0 (0)	0 (0)	<0.0001	23 (88.4)	3 (11.6)	0.0011
Low	3 (25)	9 (75)	0 (0)		10 (83.3)	2 (16.6)	
High	0 (0)	3 (25)	9 (75)		4 (33.3)	8 (66.6)	

PD-L1, programmed death ligand 1; TIL, tumor-infiltrating lymphocytes.

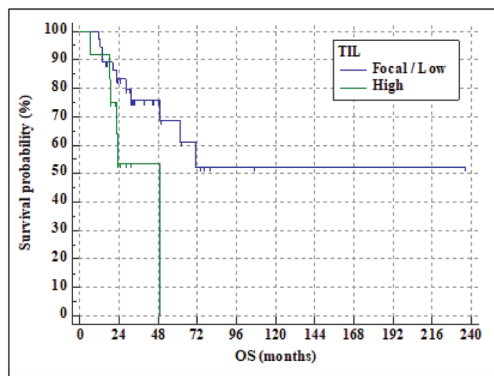


Figure 3. Kaplan Meier curve showing correlation between TILs (<50% versus >50%) and OS.

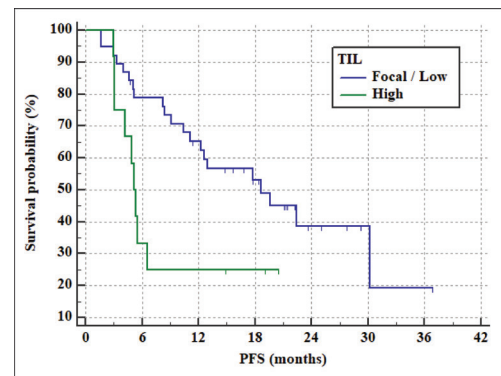


Figure 4. Kaplan Meier curve showing correlation between TILs (<50% versus >50%) and PFS.

assays, and interpretative uncertainties (e.g., cut off for positivity).

Blockade of the PD-L1/PD-1 pathway is a new promising therapeutic approach in oncology. We investigated the associations between TIL count/PD-L1 expression in invasive breast cancer and a number of clinicopathologic characteristics, including prognosis.

Our data reveal that PD-L1 expression was significantly associated with elevated TILs, and point to the critical role of local immunity in limiting tumor progression. This association is supported by other studies that observed the same results using in-vitro methods (18,19) and with previous studies suggesting that the PD-L1/PD-1 pathway plays a major role in the induction and maintenance of immunological exhaustion in the breast tumor microenvironment (20,21).

In our study, high TIL counts and PD-L1 positivity (on both TILs and tumor cells) were most significantly associated with ER-/Her2+ and TNBC. This result is supported by the results of previous studies and meta-analyses that stated that PD-L1 was most significantly associated with the triple-negative high grade tumor subtype, estrogen receptor (ER) negativity and Her2 positivity (7,11,22-24).

We observed a lower level of TILs and PD-L1 expression in ductal carcinomas compared with lobular carcinomas, regardless of other pathological variables. This was in lieu with a recent study by Doğukan et al (25), while it was in contrast with a previous study that found that TILs and PD-L1 were more expressed in ductal carcinomas than lobular breast cancers (18). We have no justification for this predilection.

As regards the survival correlation, we found a trend towards better OS and PFS for patients with focal TIL density (<5% infiltration) in the whole tumor population ($P=0.0585$ and $P=0.0533$ respectively) compared to tumors with 5-50% and tumors with >50% TIL infiltration. The lack of significance may be due to the small number of patients included in our study. This trend became statistically significant when we grouped the patients into two subgroups (instead of three subgroups), less than and greater than 50% infiltration ($P=0.238$ and $P=0.0262$ respectively). This was different from what was stated in a recent systematic review and meta-analysis that showed that in TNBC, patients with high TIL had better OS and DFS than patients with low TIL. Studies in this meta-analysis had different definitions for 'high' TIL infiltration, yet most of the studies defined 'high' as >50-60% TIL infiltration. This study even showed that with each 10% increase in percentage of TILs, patients with TNBC had an increased pathological complete response in patients receiving neoadjuvant treatment (26).

Regarding the relation between TIL infiltration and intrinsic subtypes, and their association with the patients' survival, the only significance we found was improved PFS for patients with focal (<5%) TIL infiltration, compared to higher counts of infiltration (>5%), only in patients with ER+/Her2- patients. This improvement was also found regarding the OS in the same population group but was not statistically significant.

This was in agreement with a recently published study by Kurozumi et al, that stated that TIL expression correlated with ER status and tumor proliferation. In this study, high TIL expression was a poor prognostic

Table 3. The effect of PD-L1 expression and TIL status on OS and progression free survival

Survival	TIL		P value	PD-L1 on immune cells			P value	PD-L1 on tumor cells		P value
	<50%	>50%		Focal	Low	High		Negative	Positive	
Median OS (months)	Not reached	48.9	0.0238	Not reached	Not reached	48.9	0.308	70.8	61.5	0.8871
Median PFS (months)	18.6	5.1	0.0262	19.6	10.4	5.5	0.3298	18.6	5.5	0.1298

PD-L1=programmed death ligand 1; TIL=tumor-infiltrating lymphocytes; OS= overall survival; PFS= progression free survival.

marker in ER-positive patients but was a good prognostic marker in ER-negative patients. In this study, TIL grades were categorized into low (<10%), intermediate (≥ 10 and $\leq 40\%$) and high ($>40\%$) groups. Therefore, Kurozumi et al concluded that the biological association between TILs and primary breast tumors may differ between ER-positive and ER-negative breast cancer (27).

These results were in contradiction to what Sobral et al found, where in their study, TILs infiltration showed no association with breast cancer specific survival (BCSS) in ER+/Her2- and Her2+ tumors, while high TIL status was significantly associated with better BCSS in only TNBC tumors (7). As regards the PD-L1 expression on tumor cells and TILs, we did not find any statistically significant correlation between its positivity, and the survival of the patients (OS and PFS). This non-significance may be also attributed to the low number of patients in our study.

These non-significant results were also found in a previous trial, that found that PD-L1+ tumor co-expression showed no correlation with survival of the patients (7).

A meta-analysis by Zhang et al, found that PD-L1 over-expression was associated with shorter OS in patients with breast cancer. This was attributed to the finding that in this meta-analysis, higher PD-L1 expression was linked to patients with poor clinico-pathologic criteria; as positive lymph nodes, higher histologic grades and ER negativity. The same results were also confirmed by another meta-analysis, that also demonstrated that PD-L1 over-expression was significantly associated with positive lymph node metastasis, poor nuclear grades, and ER negativity in breast cancer patients (28).

In a large previously mentioned meta-analysis by Huang et al, PD-L1+ tumor cells were significantly associated with shorter disease-free survival and OS (23).

A recent Chinese study found that PD-L1 positive tumor cells (PD-L1+ tumor) in TNBC patients had significantly longer DFS and OS values than those of PD-L1 negative tumor cells ($P=0.046$; $P=0.019$ respectively) (29).

In another recent meta-analysis, positive PD-L1 on TILs was associated with better survival of all patients in the pooled analysis, especially TNBC basal subtype, while positive over-expression of PD-L1 on tumor cells had a negative impact on survival of all patients especially also the TNBC basal like subtype (30).

Conclusion

There is a major controversy about the prognostic value of TIL and PD-L1 in breast cancer. TNBC and Her2 enriched are the most common types associated with TIL infiltration and PD-L1 positivity (on tumor cells and TILs), and this renders them more immunogenic than other subtypes. This is especially important in TNBC because of the lack of any targeted therapeutic solution other than chemotherapy. Further studies are warranted to determine the predictive value of PD-L1 positivity and TIL infiltration regarding the response to immunotherapy.

Limitations of the study

Our study included 50 patients, which could be of relatively limited sample size. Although the results reached a statistically significant correlation between PDL1 expression and TNBC, yet it did not correlate with patients' PFS and OS, which could be attributed to the limited number.

Authors' contribution

MMD: Collection and analysis of data, extraction of data from patient records, design research methodology AYR, DAS, AEE, DEMS: idea and study plan, supervision, revision of data analysis and manuscript revision MMS: revision of pathology reports and immunostains, scoring, counting and photographing of TIL, PDL1 immunohistochemical expression, supervision, revision and submission of the manuscript. All authors have read and approved the content of the manuscript and confirmed the accuracy or integrity of any part of the work.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The institutional ethical committee at Ain Shams University, Faculty of Medicine approved all study protocols (IRB approval number; MD 271/2018). The study was based on data collection and immunohistochemical analysis of positively charged slides prepared from paraffin blocks. Accordingly, informed consent was obtained at their admission time. This study was extracted from M.D thesis of Mohamed Mostafa Darwish at this university. Additionally, ethical issues (including plagiarism, data fabrication, double publication) were completely observed by the authors.

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