

Research Article

Expression of Programmed Death Ligand-1 and Correlation with Clinicopathological Features and CD8 Infiltration in Breast Cancer

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Abstract

Background: Breast cancer (BC) is considered one of the most diversified types of tumors, characterized by a high mutational burden in the tumor milieu and a lack of immune cell makeup. The programmed death receptor-1 (PD -1)/programmed death ligand-1 (PD -L1) axis has been identified as a new target in the field of immunotherapy because, when activated, they worsen the future scenarios of the disease by helping tumor cells (TC) to escape immune surveillance. This study aims to investigate the expression of PD-L1 in BC tissues from Sudanese ladies and correlate its expression with clinicopathological features and the infiltration of CD8+T lymphocytes by immunohistochemistry (IHC).

Methods: One hundred and fifty archived BC blocks were collected from National Public Health Laboratory from January 2019 to August 2020. Data regarding age, TNM staging, grade, and hormonal status were considered. Tissue sections were examined using IHC to determine the expression of PD-L1 and CD8.

Results: Among one hundred and fifty BC samples, 73 (48.7%) were TNBCs, and 77 (51.3%) were hormone-positive BCs. PD-L1 was significantly associated with BC subtypes, especially TNBCs ($P = 0.001$), a similar significant association was shown with CD8 infiltration ($P = 0.006$). None of the clinicopathological features were associated with PD-L1 expression.

Conclusion: PD-L1 expression is strongly associated with TNBC's and linked to CD8+ cells infiltration to the tumor milieu. Moreover, no correlation has been observed between the expression of PD-L1 and clinicopathological features in this study.

Keywords: immune therapy, PD-1, PD-L1, TILs infiltration, TNBCs, immune-check points blockers

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1. Introduction

Breast cancer (BC) treatment has significantly advanced in recent years, allowing for the creation of tailored and targeted therapies that delay the spread of the illness and prolong patient lives. For the triple-negative population lacking focused therapy choices, there is still a demand for novel therapeutic options for dispersed or invasive diseases [1].

Cancer immunotherapy has lately been a key source of research, notably for immune checkpoint targeted therapy, such as cytotoxic lymphocyte-associated antigen-4 (CTLA-4) and the programmed death receptor-1 (PD-1)/programmed death ligand-1 (PD-L1) axis, as well as breast cancer susceptibility gene 1 (BRCA1) [2]. The PD-1's primary ligand, PD-L1, can cause tumor-related immune cells to commit apoptosis; hence, it is considered to take part in cancer immune evasion in the tumor milieu [2].

As BC is considered to be one of the most heterogeneous types of cancer that may result in variations within the tumor-immune milieu composition and the impact of immune checkpoint inhibitors, five intrinsic subtypes of BC have been identified as a result of gene expression profiling: luminal A and B, HER2-rich, claudin-deficient, and basal-like (which is frequently used synonymously with TNBC in the literature) [2, 3]. The incidences of these subgroups have been reported to differ along with outcomes, and responses to chemotherapy, and this makes them a potential target for studies of immune-check points therapies, the immune therapies [3].

Programmed death-1 is abundantly expressed on activated monocytes, dendritic cells, B cells, natural killer cells, and cytotoxic effector T-cells (CD4 and CD8); whereas its ligands (the PD-L1 and PD-L2) can be expressed by normal and TCs as well as other cells in the tumor microenvironment such as tumor-infiltrating lymphocytes (TILs), macrophages, and fibroblasts [4].

Physiologically, T-lymphocytes become less active and competent to react to invading antigens when PD-1 binds to its ligands, and this contributes to the prevention of immune cell attacks on normal cells; hence, the description "immune-check points" comes to the surface. In the context of cancer, this physiological protective mechanism causes TCs to evade the immune system's recognition and subsequent elimination [2].

According to theories, either oncogenic processes [4] or activated tumor antigen-specific T cells that release interferons like CD8⁺ [5] can cause TCs to express PD-L1 constitutively. Interferon stimulates and tightly modulates the PD-L1 gene, and it has been seen that interferon and other inflammatory genes are linearly correlated with PD-L1 transcript expression in BC [6].

According to the data, the responses to the PD-1/PD-L1 pathway inhibition may depend largely on inducible PD-L1 expression, in this situation, responses to anticancer necessitate the pre-existence of PD-1-positive T-cells with tumor antigen specificity [4].

Moreover, various studies have correlated increased PD-L1 protein/mRNA expression and number of TILs on the tumor side [7-10]. Hence, TILs and tumor PD-L1 overexpression may be functionally related [10]. On the other hand, PD-L1 expression has been strongly correlated with unfavorable clinicopathologic characteristics of BC [11].

In the summary of the meta-analysis study by Huang *et al.*, it was observed that PD-L1 positive expression was significantly related to ductal carcinoma, size of the tumor, grade 3 tumors, ER negative, PR negative, TNBCs along with TILs, but not with patient age or lymph node metastases [12]; and there were many different conclusions on the prognostic and/or predictive relevance of PD-L1's expression in BC [13]. This diversity may be justified by the heterogeneity of PD-L1's expression along different antibody clones, spatial expression (cytoplasmic or membranous), cell type expressing this marker (TILs versus TCs), and tissue type used whether it is TMA or whole tissue [12, 13].

In this study, we are correlating PD-L1 expression to molecular types of BC and assessing the relevance and significance of the co-expression of PD-L1 and CD8+ TILs.

2. Methods

This retrospective case study aims to study the expression of PD-L1 and CD8+ T lymphocyte infiltration by immunohistochemistry (IHC) in BC tissues and to correlate PD-L1 expression with clinicopathological characteristics.

One hundred and fifty archived BC blocks have been collected from STAK, the National Public Health Laboratory, from January 2019 to August 2020.

The formalin fixed paraffin embedded BC blocks were sectioned in 3 consecutive paraffin sections each of 3 μ m thickness, one of the sections was stained with hematoxylin and eosin to reaffirm diagnosis, and the rest of the sections were picked onto a charged slide for subsequent IHC demonstration with anti-CD8 and anti-PD-L1.

Data considering age, histological diagnosis, histological grading, lymph node involvement, tumor size and metastasis, the clinicopathological features along with data describing hormonal status (Estrogen (ER), Progesterone receptors (PR), and human epidermal growth factor receptor-2 (HER2)) were also considered and gathered.

Anti-CD8 mouse monoclonal antibody (32-M4- DAKO), and anti-PD-L1 mouse monoclonal antibody (73-10, Abcam, UK) were used to raise immune-staining. Positive PD-L1

expression in the cytoplasm and/or cellular membrane was considered, this positivity has been counted and scored based on a system that counts the percentage and intensity of positivity utilizing the Histo-scoring system (H-score), the system which classifies cells down to staining intensity and cellular density. A cut-off point of 100 was used to divide the expression into two groups: (0-99 as negative; 100-300 as positive expression) [14].

Data analysis was done using SPSS-version 20 and Graph-Pad Prism 8 was used for graphing. Statistical tests such as frequencies, Crosstabulation, Chi-square, Mann-Whitney, and Kruskal-Wallis were used. In all the analyses done, a two-tailed *P* value of .05 was deemed statistically significant.

3. Results

Among one hundred and fifty BC samples, 73 (48.7%) were TNBCs, and 77 (51.3%) were hormonally positive BCs. The median age was 50 years (range: 23-80 years). Females aged 40-59 years were predominantly affected by BC, constituting 51.3% of the total population, while younger women (less than 40 years) and older women (more than 60 years) were the least affected, constituting 23.3% and 25.4% respectively.

Invasive ductal carcinoma of the breast was the most prevalent histologic type (81.3%), with stage I tumors being the most prevalent (46.0%), and grade III tumors being the next most frequent (47.3%). Regarding types of BC, luminal A was the most prevalent molecular subtype (84.8%). Table 1 presents frequencies and significance levels of association with PD-L1 expression.

The immuno-histochemical labeling describing the positive rates of PD-L1 in TC was 30% (45/150); this expression was anticipated to be caused by flooding of the tumor microenvironment with TILs, particularly CD8 positive cells, the crucial component in induced expression of PD-L1. Consequently, CD8 was one of the most intriguing targets for IHC analysis to ascertain whether there is a connection between CD8 and PD-L1 expressions. Table 2 demonstrates a statistically significant correlation between PD-L1 expression and CD8 infiltration at the tumor location *P* value (< .05).

Regarding the mode of distribution of PD-L1's scores among different categories of BC, we performed the Kruskal-Wallis test to explore the allocation of PD-L1 scores among BC molecular subtypes highlighted by hormonal expression patterns in luminal A, luminal B, Her2 enriched, and triple-negative BC.

Table 3 elaborates on diverse PD-L1 distribution among molecular subsets of BC, given that the cut-off point of 100 is taken as a point to govern PD-L1 negativity or

positivity through the H-score ranges (0-300). The test found a significant diversity in distribution between molecular subtypes of BC in ($P = 0.011$).

Similarly, this score distribution dissimilarity was double highlighted in BC samples when simply classified as TNBCs and non-TNBCs regardless of detailed luminal status. Mann-Whitney test was applied to evaluate the variation of PD-L1 score distribution among the two groups of BC, the triple negative, and their hormonal positive counterparts. In Figure 1, there was an obvious variable distribution of PD-L1 scores across TNBCs and non-TNBCs.

In addition, closely related result finding was addressed to BC types from a histological point of classification, we found that there was a significant dissimilar distribution of PD-L1's scores across IDC, ILC, and other tumor types like papillary carcinoma, and intraductal hypersecretory breast carcinoma ($P = 0.028$), this is well illustrated in Table 4.

TABLE 1: Characterizes the distribution of clinicopathological features of BC and its association with PD-L1 expression.

Clinicopathological data	Frequency	Percentage (%)	P-value
PD-L1:			
Positive	45	30	–
Negative	105	70	
Age group (yr):			0.36
≤39	35	23.3	
40–59	77	51.3	
≥60	38	25.3	
Stage:			0.34
I	69	46.0	
II	27	18.0	
III	53	35.3	
IV	1	0.7	
Grade:			0.58
I	15	10.0	
II	64	42.7	
III	71	47.3	
Tumor type:			0.028
IDC	122	81.3	
ILC	6	4.0	
Others	22	14.7	
Total	150	100%	

TABLE 2: The correlation between PD-L1 and CD8.

		PD-L1		P-value = 0.006
		Negative	Positive	
CD8	Negative	49 (83.1%)	10 (16.9%)	
	Positive	56 (61.5%)	35 (38.5%)	
Total		105	45	

TABLE 3: Distribution of PD-L1 across molecular subtypes of breast cancer.

	PD-L1		
	Negative	Positive	
BC subtypes	(Score is less than or equal 99)	(Score is more than or equal to 100)	
Luminal A	39 (84.8%)	7(15.2%)	
	Total = 46		P-value = 0.011
Luminal B	16 (76.2%)	5(23.8%)	
	Total = 21		
HER2 enriched	9 (90.0%)	1(10.0%)	
Total = 77	Total = 10		
Triple negative BC	41 (56.2%)	32 (43.8%)	
Total = 73	Total		
	105	45	

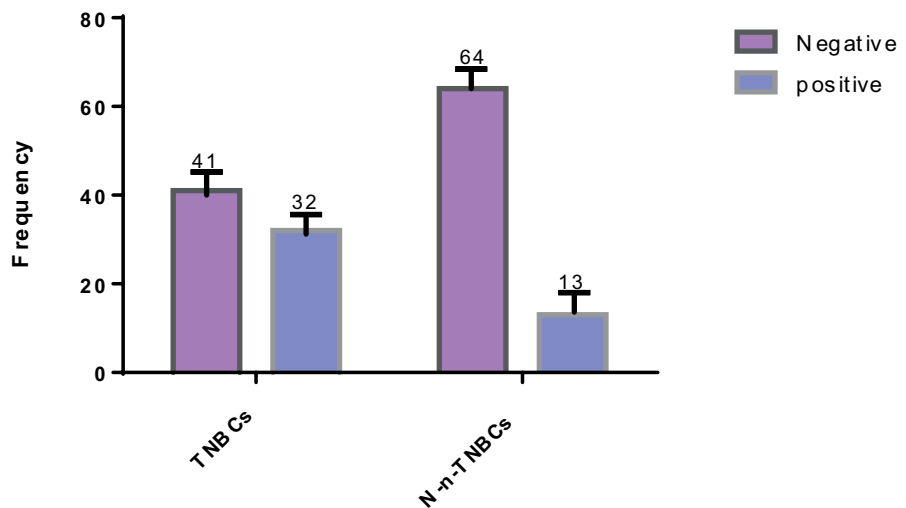


Figure 1: The difference in PD-L1 distribution between TNBCs and non-TNBCs.

TABLE 4: Expression of PD-L1 across histological types of breast cancer using Kruskal–Wallis test.

	PD-L1		
	Negative	Positive	
BC subtypes	(Score is less than or equal 99)	(Score is more than or equal to 100)	
IDC	45 (62.5%)	27 (37.5%)	P-value = 0.028
ILC	33 (86.8%)	5 (13.2%)	
Others	27 (67.5%)	13 (32.5%)	
Total	105	45	

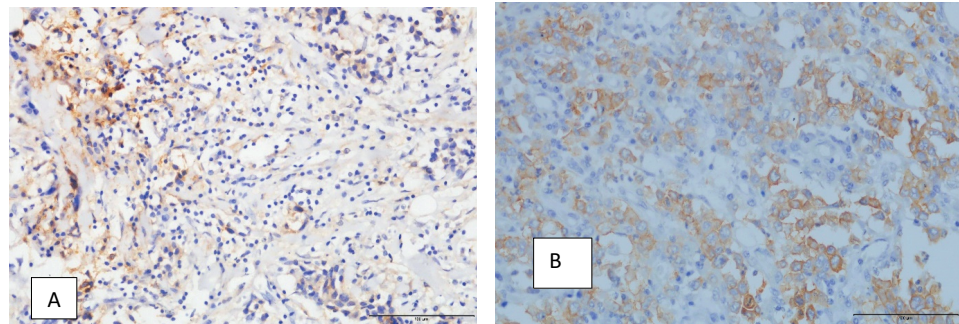


Figure 2: (A) Expression of PD-L1 in tumors cells. (B) Tumor cells expressing PD-L1 and immune cells.

4. Discussion

Of all cancers affecting women globally, BC has the greatest incidence and fatality rates, it has been anticipated by GLOBOCAN that by the year 2040, the incidences in Africa may virtually be doubled from the meantime [15].

Little is known about incidences in Sudan but according to data from the Khartoum State Cancer Registry for the years 2009 to 2010, BC was the most prevalent type of cancer among Sudanese women [16]. This dictates rapid and intensive searches in different treatment regimens that eradicates cancer safely with few side effects, hence immune-therapeutic options have dragged attention and rapid attempts to examine different populations from different ethnicities.

PD-L1 as predicting marker for subsequent immune-check blockers, was subjected to association tests with the age group of BC's patients along with the clinicopathological characteristics stage, grade, and molecular subtypes.

In this study, PD-L1 was expressed in rates of 30% which seems to be in a reasonable range among studies held on whole tissue section [11, 17, 18]. In general terms, there is a considerable discrepancy between rates of PD-L1 expression as there is no standardized method for scoring beside variable cut-off points for PD-L1 positivity [12].

In the current study, we found that there is an insignificant link between the age of patients and PD-L1's expression, this comes in concordance with a conclusion by Ayoub *et al.* and Lou *et al.* while when it comes to the term of BC's grade and stage, we found no link between any and PD-L1 expression unlike what was concluded by researchers in the same studies [11, 19]; moreover, data from cBioPortal-TCGA, PanCancer Atlas that retrieved from 1082 patients' samples diagnosed with invasive breast carcinoma, presented that the correlation between age and PD-L1 expression at the level of mRNA (*CD274* gene mRNA expression) was insignificant indicating that expression of PD-L1 is not linked to how young or old is the subject, in other words, age is not a factor that

underlines the level of expression, and this supports the same conclusion we made around the variable [20, 21].

In addition, we utilized data pooled in the OncoDB database (<https://oncodb.org>) to compare our clinical findings of age and stage with correlation analysis based OncoDB between *CD274* gene expression, age, and pathological stage. OncoDB is a web-based tool for investigating aberrant patterns in gene expression as well as viral infection that are linked to clinical parameters in cancers [22]. The database analysis spotted neither age nor stage having any significant link to PD-L1 expression (P-values 8.3e-01 and 8.6e-02 respectively) [22], our findings also go in concordance with this web-based analysis.

Regarding differences in PD-L1 expression/distribution between histological types of BC, we found significant and variable distribution between IDC, ILC, and other tumors with predominance in IDC, this result is supported by reports from Kassardjian *et al.* who found the only positive PD-L1 expression was in 4.1% IDC versus negative/absent expression in the remaining tumors [23], likewise, data from cBioPortal-TCGA, PanCancer Atlas graphically reveals that *CD274* gene/PD-L1 expressions are more accumulated in IDCs than other types of breast tumors [20, 21].

Additionally, PD-L1 expression was significantly obvious in TNBCs versus non-TNBC tumors, a finding that is supported by Qin and his colleagues [24, 25]. On the same level, the minute differences in the distribution of PD-L1 positivity among the molecular subtypes of BC, TNBCs, luminal types, and Her2 enriched type were found to be significant in concordance with data from the Cancer Genome Atlas and conclusions from many other studies [24, 26-28].

A strong positive association between CD8-positive tumors and PD-L1 expression points to the pivotal role of TILs infiltration in the tumor microenvironment and expression of PD-L1, our finding is consistent with what has been reported in many studies [29]. And according to Shenasa *et al.* who concluded that TILs has no unusual predictability for gaining substantial benefits from chemotherapy but still can tell that the primary molecular subgroups, the non-luminal, and basal BC subtypes are frequently willing to immunotherapy [30]. In general terms, TILs like CD8, CD4, B cells, and macrophages were found to be associated with favorable outcomes of the disease [28] (27).

On the other hand, mRNA gene expression correlations of *CD274* gene (PD-L1 protein) and CD8+lymphocytes gene *CD8A* are the mRNAs that are hugely considered as the role players controlling subsequent protein expression steps and lead the translation in the tumor microenvironment. Moreover, we once more looked at the data provided in the cBioPortal-TCGA, PanCancer Atlas from the aspect of mRNA expressions of *CD274*

and *CD8a* genes, and found significant links at the level of protein expression and TIL infiltration. We also highlighted in our study that TCGA data conclusions state strong positive correlation between two genes at the mRNA level (their p-value = 9.26e-88) [20, 21].

5. Conclusion

To the best of our knowledge, this study is the first to illustrate PD-L1 expression in Sudanese women with BC. As shown in this study, the distribution of PD-L1 expression in TCs varied between molecular subtypes of BC with an obvious predominance in TNBCs, the most aggressive type of BC, and this predicts a promising response to the newly invented treatment regimen.

Hence, to determine whether patients may gain from PD-1/PD-L1 checkpoint blockade therapy, it is necessary to deep dive into the tumor microenvironment to study the pattern of PD-L1's expression amongst the broad subtypes of BC.

On the other hand, the absence of a significant correlation between PDL-1 expression and clinicopathological features seen in this study necessitates further studies attempting to elaborate this association considering the demographic data from Sudanese BC to make concrete for further advanced studies exploring mutational levels in the tumor milieu.

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Ethical Considerations

The Sudan University of Science and Technology's institutional ethics committee gave its approval to this work (reference number for the ethical committee is DSR-IEC-05-08). Since most patients passed while survivors had no contact information, it was difficult to obtain their consent. Hence to protect patients' privacy, all samples and medical data utilized in this study have been securely anonymized.

Competing Interests

No competing interests were disclosed.

Availability of Data and Material

The dataset generated during this study are available from the corresponding author on reasonable request.

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