RESEARCH ARTICLE

PD-L1 and CD8+ T cell evaluation in breast cancers and their correlation with clinicopathological parameters

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Abstract

Objective: To determine the immunohistochemical expression of Programmed cell Death Ligand 1 and intratumoural cluster of differentiation-8-positive T lymphocyte count in primary breast cancer cases, and to ascertain their association with different clinicopathological parameters.

Method: The cross-sectional study was conducted at the Pakistan Navy Station Shifa Hospital, Karachi, from January 2020 to December 2021, and comprised patients of breast cancer regardless of age. Representative tissue blocks, both prospective and from the 2019 institutional archives, were exposed to immunohistochemical staining with Programmed cell Death Ligand 1 and intratumoural cluster of differentiation-8-positive T lymphocyte antibodies. Pathological and clinical records were used for assessing clinicopathological parameters. Data was analysed using SPSS 23.

Results: Of the 70 women with mean age 52.04±12.54 years, 30(42.9%) expressed high Programmed cell Death Ligand 1 positivity, and 55(78.6%) revealed low intratumoural cluster of differentiation-8-positive T lymphocyte count, while 23 (32.9%), had both Programmed cell Death Ligand 1 high positivity and low intratumoural cluster of differentiation-8-positive T lymphocyte count. The association between Programmed cell Death Ligand 1 and intratumoural cluster of differentiation-8-positive T lymphocytes was not significant (p=0.813). A strong significant association was observed between Programmed cell Death Ligand 1 expression and progesterone receptor negative status (p=0.008). No significant association was observed with any other clinicopathological parameter.

Conclusion: Programmed cell Death Ligand 1 high positivity and low intratumoural cluster of differentiation-8-positive T lymphocyte count were together observed in one-third of the breast cancer cases. A strong significant association existed between Programmed cell Death Ligand 1 high positivity and progesterone receptor negative status.

Keywords: Breast cancer, Programmed cell death 1 ligand 1 protein human, CD8 positive T lymphocytes, Immunohistochemistry. (JPMA 74: 1274; 2024) **DOI: https://doi.org/10.47391/JPMA.10567**

Introduction

Breast cancer (BC) universally is the predominant malignancy, with incidence in both genders collectively at 11.7%.[1] It is also emerging to be the most extensive malignancy in adults in Pakistan,[2] with an incidence of 14.5%.[1] Pakistan has a colossal load of new BC cases in conjunction with the prevailing cases.[2]

BC is a diversified disease influenced by both genetics and hormones.[3,4] The surrounding tumour microenvironment has now been established to be a critical aspect of any tumour[3] and one of the major constituents of this environment are the cluster of differentiation (CD) 8-positive T (CD8+T) lymphocytes, which, to function, rely on co-stimulatory and co-inhibitory signals.[5] The co-inhibitory signals, or immune checkpoints, halt the over-activation of T lymphocytes, and, thus, hinder autoimmunity.[6] One such co-inhibitory receptor is the Programmed cell Death protein (PD-1),[7] which is expressed by activated lymphocytes, and binds to its ligand Programmed cell Death Ligand 1 (PD-L1), thereby inhibiting cytotoxic T cells' function and preventing their overstimulation.[5] The expression of PD-L1 has been noted in many areas of the body, and can be detected in variable forms.[8] Malignant cells, which include, but are not limited to, melanomas, colorectal, breast and ovarian, also express PD-L1 and thereby escape the immune response by inhibiting intratumoural T lymphocytes, enhancing tumour cell proliferation.[9]

Over the years, the PD-1/PD-L1 pathway has gained huge popularity for its function in BC progression, particularly because of the significant potential role of immunotherapeutic treatment.[8,10] Anti-PD-1 and anti PD-L1 drug trials have already been undertaken, and many have demonstrated a favourable response in BC management.[3,10] Not enough research has been conducted in Pakistan regarding the expression of PD-L1 and intratumoural CD8+T lymphocyte count and their association with the various clinicopathological parameters in BC patients. The current study was planned to fill the gap in literature by determining the immunohistochemical

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(IHC) expression of PD-L1 and intratumoural CD8+T lymphocyte count in primary BC cases, and to ascertain their association with different clinicopathological parameters.

Patients and Methods

The analytical, cross-sectional study was conducted at the Pakistan Navy Station (PNS) Shifa Hospital, Karachi, from January 9, 2020, to December 30, 2021. After approval from the ethics review committee of Bahria University Medical and Dental College (BUMDC), Karachi, the sample size was determined using Open Epi Version 3[11] with the formula; n=[DEFF*Np(1-p)]/ [(d2/Z21-α/2*(N-1)+p*(1-p)], taking 95% confidence interval (CI) and 5% confidence limit, while BC prevalence was taken from an earlier Pakistani sample.[12] The sample was raised using non-probability convenience sampling technique. The formalin fixed, paraffin embedded (FFPE) blocks of histologically diagnosed BC specimens from prospective cases were selected along with histopathological archives of 2019.

The cases included were primary pre-treatment BC specimens regardless of patient age, and had known status for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Any specimen that was inadequate or poorly fixed was excluded. Patient consent had been taken prior to biopsy.

After the review of the Haematoxylin and Eosin (H&E) slides by two histopathologists, IHC was performed. The FFPE blocks were sectioned into 3-5µm, placed on poly-L lysine-coated slides, and fixated at 80ºC for 20-25min. Deparaffinisation, hydration and antigen retrieval was achieved through a pretreatment system (Dako PT Link PT200, Denmark) in 45-60min, and blocking of endogenous peroxidase was performed using hydrogen peroxide (H_2O_2) solution for 10-15min. Primary antibodies PD-L1 and CD8 were then applied after dilution in the ratio of 1:100 as per the manufacturer's protocol. Briefly, after applying the antibody to cover the section, the sections were incubated in a humidity chamber at room temperature for 45-60min. After washing twice with a buffer solution, an enhancer was applied for 10-15min after which it was washed off. The secondary antibody was horseradish peroxidase (HRP) which was then applied to the slides for 35-45min at room temperature, followed by diaminobenzidine (DAB) substrate chromogen for 1-2min and washed off. The slides were counterstained with Haematoxylin, and after mounting in toluene-free mounting media (Dako, code no.CS705, Denmark), they were observed under a light microscope (Nikon YS 100, code no. 2CE-MTNH-6, Japan).

PD-L1 monospecific mouse monoclonal antibody (ZM-100, product ID Z2519MS) and CD8 mouse monoclonal antibody (ZM-54, product ID Z2364MS) (Zeta Corporation, United States) were both localised on membrane. Placenta and lymph node (LN) were used as positive control tissues for PD-L1 and CD8, respectively.

For PD-L1 interpretation, the staining was determined to be either negative (no staining), low positive (1-49% staining) or high positive (≥50% staining) in tumour cells. Membranous staining was taken into account, while cytoplasmic and intensity of staining were ignored.[13]

For intratumoural CD8+ T lymphocyte count, two groups were created on the average of 5 randomly selected high power fields (HPFs). Initial CD8+T lymphocytes were recognised as + (1-25 cells), ++ (26-50 cells), and +++ (>50 cells) in the tumour nest. Then they were finally categorised as low (≤25 cells) or high (>25 cells). Stromal tumour infiltrating lymphocytes (TILs) were ignored.[4,14]

Data was analysed using SPSS 23. Continuous variables were presented as mean±standard deviation, whereas categorical data was expressed as frequencies and percentages. Pearson's chi-square test and Fisher's exact test were used to assess the association of PD-L1 and intratumoural CD8+T lymphocytes, as well as their association with various clinicopathological parameters. P<0.05 was taken as statistically significant. The correlation coefficient (*r*-value) was also calculated, where appropriate, for continuous variables to determine the strength of associations between them.

Results

There were 70 women with mean age 52.04±12.54 years. The specimens were acquired via 3 modes; modified radical mastectomy (MRM) 26(37.1%,), Trucut biopsy 39(55.7%), and lumpectomy 5(7.1%) cases. The majority of cases 64(91.4%) were of invasive ductal carcinoma (IDC/invasive carcinoma of no special type (NST), with 62(88.6%) having grade 2, and 12(54.5%) with stage 2. The luminal subtype was the most common 38(60%) cases, and 38(60.3%) ER-positive cases, 38(60.3%) PR-negative cases and 33(52.4%) HER2-positive cases were observed. Overall, 30(42.9%) cases expressed high PD-L1 positivity, and 55(78.6%) revealed low intratumoural CD8+T lymphocyte count,

Table-1: Comparison of PD-L1 expression and CD8+ T cell count in breast cancer cases (n=70).

	Low CD8+ T cell count	High CD8+ T cell count	Total n (%)	*p*-value	*r*-value
	n (%)	n (%)			
Negative PD-L1 expression	20 (28.6%)	4 (5.7%)	24 (34.2%)		
Low Positive PD-L1 expression	12 (17.1%)	4 (5.7%)	16 (22.9%)	0.813	0.068
High Positive PD-L1 expression	23 (32.9%)	7 (10%)	30 (42.9%)		
Total	55 (78.6%)	15 (21.4%)	70 (100%)		

Table-2: Comparison of PD-L1 expression and CD8+ T cell count with clinicopathological parameters in breast cancer cases (n=70).

Parameter	n (%)	PD-L1 expression [n (%)]					CD8+ count [n (%)]			
		Neg	Low +	High+	*p*-value	*r*-value	Low	High	*p*-value	*r*-value
Age group (years)	n=70	n=24	n=16	n=30	0.835	0.114	n=55	n=15	0.547	0.086
20-29	2 (2.9)	1 (50.0)	0	1(50.0)			2 (100)	0		
30-39	9 (12.9)	4 (44.4)	2(22.2)	3(33.3)			8 (88.9)	1(11.1)		
40-49	17 (24.3)	6 (35.3)	5 (29.4)	6(35.3)			12(70.6)	5(29.4)		
50-59	16 (22.9)	4 (25.0)	6 (37.5)	6(37.5)			13(81.3)	3(18.7)		
60-69	22 (31.4)	7 (31.9)	3(13.6)	12(54.5)			17(77.3)	5(22.7)		
70-79	3 (4.2)	2 (66.7)	0	1(33.3)			3 (100)	0		
80-89	1 (1.4)	0	0	1(100)			0	1(100%)		
Histopathological variant	n=70	n=24	n=16	n=30	0.174	-	n=55	n=15	0.373	-
Invasive ductal carcinoma/ Invasive carcinoma NST	64 (91.4)	23(36.0)	16 (25.0)	25(39.0)			50(78.1)	14 (21.9)		
Invasive lobular carcinom	4 (5.7)	0	0	4(100)			4(100%)	0		
Invasive ductal carcinoma with neuroendocrine differentiation	2 (2.9)	1(50.0)	0	1(50.0)			1(50.0%)	1(50.0%)		
Grade	n=70	n=24	n=16	n=30	0.885	0.040	n=55	n=15	0.388	0.162
1	3 (4.3)	2(66.7)	0	1 (33.3)			3(100)	0		
2	62(88.6)	20(32.3)	15(24.2)	27 (43.5)			49(79.0)	13(21.0)		
3	5 (7.1)	2(40.0)	1 (20.0)	2(40.0)			3(60.0)	2(40.0)		
Stage	n=22	n=8	n=7	n=7	0.596	0.090	n=17	n=5	0.843	0.089
1	4(18.2)	2 (50.0)	0	2(50.0)			3(75.0)	1(25.0)		
2	12 (54.5)	5 (41.7)	4(33.3)	3(25.0)			10(83.3)	2(16.7)		
3	6 (27.3)	1 (16.7)	3(50.0)	2(33.3)			4(66.7)	2(33.3)		
Molecular Subtype	n=63	n=21	n=14	n=28	0.376	-	n=49	n=14	0.092	-
Luminal	38 (60)	16(42.1)	6(15.8)	16(42.1)			27(71.0)	11(29.0)		
HER2 Enriched	13 (21)	3(23.1)	4(30.8)	6(46.1)			13 (100)	0		
Triple Negative	12 (19)	2 (16.7)	4 (33.3)	6(50.0)			9 (75)	3(25)		
Individual Hormone Receptor Status	n=63	n=21	n=14	n=28	0.128	-	n=49	n=14	0.113	-
ER Positive	38(60.3)	16 (42.1)	6(15.8)	16(42.1)			27 (71.0)	11(29.0)		
ER Negative	25(39.7)	5 (20)	8(32.0)	12(48.0)			22(88)	3(12)		
PR Positive	25(39.7)	14 (56)	3 (12)	8(32)			20 (80)	5 (20)	0.731	-
PR Negative	38 (60.3)	7 (18.4)	11 (29.0)	20(52.6)	0.008	-	29 (76.3)	9 (23.7)		
HER2 Positive	33(52.4)	10(30.3)	7(21.2)	16(48.5)	0.788	-	27 (81.9)	6(18.1)	0.418	-
HER2 Negative	30(47.6)	11 (36.7)	7 (23.3)	12 (40)			22 (73.3)	8(26.7)		
Lymph node involvement	n=70	n=24	n=16	n=30	1.098	-	n=55	n=15	0.364	-
Present	10(14.3)	3 (30	4 (40)	3 (30)			7(70)	3 (30)		
Absent	15(21.4)	4 (26.7)	6 (40)	5 (33.3)			13(86.7)	2(13.3)		
Unknown	45(64.3)	17(37.8)	6 (13.3)	22 (48.9)			35 (77.8)	10 (22.2)		
Lymphovascular invasion	n=70	n=24	n=16	n=30	0.974	-	n=55	n=15	0.663	-
Present	32(45.7)	12(37.5)	8(25)	12(37.5)			26 (81.3)	6(18.7)		
Absent	11(15.7)	3(27.3)	3 (27.3)	5 (45.4)			10 (90.9)	1 (9.1)		
Unknown/undetermined	27(38.6)	9 (33.3)	5 (18.5)	13(48.2)			19 (70.4)	8 (29.6)		

PD-L1: Programmed cell death ligand 1, CD8+: Cluster of differentiation 8-positive, NST: No special type, ER: estrogen reception, PR: Progesterone receptor, HER2: Human epidermal growth factor receptor 2.

while 23 (32.9%) had both PD-L1 high positivity and low intratumoural CD8+ T lymphocyte count. The association between PD-L1 and intratumoural CD8+T lymphocytes was not statistically significant (p=0.813, r=0.068) (Table 1).

A strong significant association was observed between PD-L1 expression and PR-negative status (p=0.008). No significant association was observed with any other clinicopathological parameter (Table 2).

Discussion

In the current study, majority of the cases displayed high positive PD-L1 expression (42.9%). This is in concordance with studies in which 51.6%, 46% and 51.7% cases revealed high expression of PD-L1.[5,15,16]

Conflicting results have also been reported, with PD-L1 positivity being only 8.1%, 23.1% and 19.4% cases.[14,17,18] The substantial variation could be attributed to the fact that PD-L1 expresses itself in various forms in the body, such as proteins, messenger ribonucleic acid (mRNA),

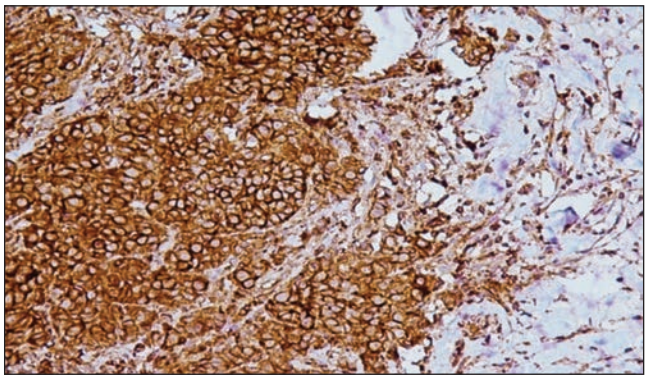

Figure-1: Immunohistochemical (IHC) photomicrograph of invasive ductal carcinoma/NST showing PD-L1 high positivity (x20)
PD-L1: Programmed cell death ligand 1, NST: No special type.

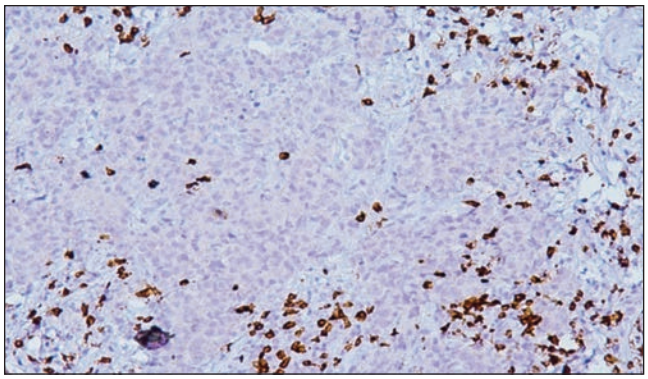

Figure-2: Immunohistochemical (IHC) photomicrograph of invasive ductal carcinoma/NST showing low intratumoural CD8+ T lymphocyte count (the same case as in Figure 1; x20).
CD8+: Cluster of differentiation 8-positive, NST: No special type.

soluble forms in the blood, exosomes and/or nuclear form.[8] Consequently, there are diverse methodological techniques available for its identification, including IHC, leading to no set criteria for its evaluation and scoring.[13,19] Additionally, sample sizes crucially differ amongst studies done across the globe.[19,20]

Most of the current cases had a low intratumoural CD8+ T lymphocyte count (78.6%), which related to a study that demonstrated low intratumoural CD8+T lymphocyte count in breast tumours in comparison to normal tissue.[19] Another study revealed a decrease in intratumoural CD8+ T lymphocyte count as the stage increased.[21] Differing evidence shows an increase in intratumoural CD8+ T lymphocytes in BC, especially in the triple-negative BC (TNBC) subset.[21,22] Similar to the scoring method of PD-L1, there is no defined threshold to assess tumour infiltrating lymphocytes (TILs) and CD8+ T lymphocytes, and different cut-off values are in usage.[4,14]

The current study demonstrated PD-L1 high positivity with low intratumoural CD8+T lymphocyte count (32.9%) (p=0.813, r=0.068), which was in concordance with studies on BC[5] and pancreatic cancer.[23] This could imply that tumour cells primarily express PD-L1 constitutively, which is based on activation pathways that are controlled genetically.[24] As PD-L1 is an acknowledged co-inhibitory signal for T lymphocytes, the results of low intratumoural CD8+T lymphocyte count propose the possibility of PD-L1-induced T cell exhaustion and apoptosis in cancer cells by different means.[25] Intriguingly, several results have reported a significant association with high PD-L1 expression and a high intratumoural CD8+T lymphocyte count.[20,24] This could, on the other hand, propose that the expression of PD-L1 by malignant cells is an adaptive response to an assertive primary immune reaction, and that intratumoural CD8+T lymphocytes are an independent prognostic factor.

In the current study, a significant association was observed between PD-L1 and PR-negative status (p=0.008), which is comparable to earlier studies.[13,14,20] Hormone receptor negative status is a known marker of aggressive tumours, and carries the poorest prognosis[20] hence, the expression of PD-L1 may be employed as a marker for aggressive cancers. The current study highlighted the possible function of anti PD-L1 drugs and the importance of immunotherapy in BC. Interestingly, no significant association was observed between PD-L1 and molecular subtypes, as well as between individual ER and HER2 status, although studies have shown a significant relation.[14,20] This could be simply due to the small sample size in the current study.

Expectedly, high PD-L1 positivity was noted more in TNBC category, concurrent to other studies.[16,19,20]

The majority of the current cases (55.7%) were of Trucut biopsies, therefore the stage, LN involvement and lymphovascular invasion (LVI) of only a few cases could be studied. Although no significant association was observed between these parameters as well as grade, age and variant with PD-L1 or intratumoural CD8+T lymphocytes, certain remarkable findings were observed. Firstly, PD-L1 high positivity with low intratumoural CD8+T lymphocyte count was seen in the age group 60-69 years. This may be due to the fact that ageing cells secrete factors, such as tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), unlike the dormant cells.[26] As TNF-α stimulates the expression of PD-L1 via the nuclear factor kappa b (NF-κB) pathway[9] this could describe the increased PD-L1 expression in the advanced age group. Moreover, aging causes a decrease in T lymphocyte formation due to involution of thymic fat and exhaustion of naïve T lymphocytes through infection in the youthful years, and the probable inhibitory effects of PD-L1,[26] leading to a lower intratumoural CD8+ T lymphocyte count. Secondly, high PD-L1 expression seen in the higher

grades in the current study as well as in literature[14,20] suggests that as the grade increases, the tumour may contain an augmented concentration of neo-antigens that may lead to an increased expression of PD-L1.[24] Lastly, the absence of LN involvement may suggest the function of PD-L1 against LN metastasis.[24] However, larger cohorts and sample sizes are needed.

The present study had its limitations, like having a small sample size taken from a single tertiary care hospital. Besides, LN involvement and LVI could not be assessed as the medical records of all cases were not available. Prospective, multicentre studies on larger sample sizes are recommended to validate the current findings.

There is a dire need to standardise PD-L1 and intratumoural CD8 detection to acquire consistent results. A national cancer registry must be established in Pakistan to get the correct BC incidence and prevalence.

Conclusion

There was high expression of PD-L1 and low intratumoural CD8+T lymphocyte count in the BC cases studied. High PD-L1 positivity was seen more frequently in TNBCs. A strong significant association was observed between high PD-L1 positivity and PR-negative status, signifying PD-L1 to be a marker for aggressive cancers.

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Author Contribution:

SK: Conceived idea, literature search, data collection and writing.
SS: Data interpretation, literature search and critical analysis.
NJ: Data interpretation and critical analysis.