

PD-L1 expression in breast invasive ductal carcinoma with incomplete pathological response to neoadjuvant chemotherapy

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Abstract

Objectives: To investigate the expression of programmed death-ligand 1 (PD-L1) in breast cancer in association with incomplete pathological response (PR) to neoadjuvant chemotherapy (NAC).

Methods: PD-L1 expression was evaluated using immunohistochemistry in post-operative, post-NAC samples of 60 patients ($n = 60$) diagnosed with breast invasive ductal carcinoma with incomplete PR to NAC, including 31 matched pre-NAC and post-NAC samples ($n = 31$). PD-L1 protein expression was assessed using three scoring approaches, including the tumor proportion score (TPS), the immune cell score (ICS), and the combined tumor and immune cell score (combined positive score, CPS) with a 1% cut-off.

Results: In the post-operative, post-NAC samples ($n = 60$), positive expression rate of PD-L1 was observed in 18.3% (11/60) of cases by TPS, 31.7% (19/60) by ICS, and 25% (15/60) by CPS. In matched samples, positive expression rate of PD-L1 was observed in 19.3% (6/31) of patients by TPS, 51.6% (16/31) by ICS, and 19.3% (6/31) by CPS in pre-NAC specimens, while it was observed in 22.6% (7/31) of matched post-NAC samples by TPS, 22.6% (7/31) by ICS, and 19.3% (6/31) by CPS. In the matched samples, there was a significant decrease in PD-L1 immunoexpression using ICS in post-NAC specimens (McNemar's, $p = 0.020$), while no significant differences were found using TPS and CPS between pre- and post-NAC samples ($p = 1.000$, $p = 0.617$; respectively). PD-L1 immunoexpression determined by TPS or CPS was only significantly associated with ER status ($p = 0.022$, $p = 0.021$; respectively), but not with other clinicopathological variables. We could not establish a correlation between PD-L1 expression and the overall survival rate ($p > 0.05$). There were no significant differences in the tumor infiltrating lymphocytes count between the paired pre- and post-NAC samples ($t = 0.581$, $p = 0.563$ or Wilcoxon's Signed Rank test; $z = -0.625$, $p = 0.529$).

Conclusion: Our findings indicate that PD-L1 protein expression in infiltrating immune cells was significantly reduced in breast tumors that developed incomplete PR following the exposure to NAC.

Keywords

breast cancer, programmed death-ligand 1, immunohistochemistry 22C3 pharmDx, neoadjuvant chemotherapy, immunotherapy, pathological response, tumor infiltrating lymphocytes, overall survival

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Introduction

Breast cancer is the most commonly occurring malignancy among women, representing nearly 25% of cancer cases diagnosed in 2020.¹ Despite the significant advances in early screening and treatment strategies, breast cancer remains the second leading cause of cancer-related mortality among women, accounting for 15% of all female cancer-associated deaths worldwide.¹ Neoadjuvant chemotherapy (NAC) is a primary therapeutic option of locally advanced breast cancer that aims to reduce tumor size prior to breast conservation surgery and to improve overall survival.² Primarily, NAC is considered for patients diagnosed with early-stage triple-negative breast cancer (TNBC).³ However, NAC has also been associated with better outcomes (satisfactory rate of complete pathological response (PR)) in pre-menopausal breast cancer patients who have estrogen receptor-positive (ER+)/Human epidermal growth factor receptor 2-negative (HER2-) receptor status.⁴ Although NAC offers several benefits,^{5,6} cancer relapse and metastasis following treatment still pose a notable threat. Thus, a better understanding of molecular mechanisms involved in cancer cell responses to therapy, and the subsequent interactions with the immune system, is required.

Programmed death-ligand 1 (PD-L1) is a transmembrane glycoprotein expressed on the membranes of a variety of tumor cells, epithelial cells, and immune cells including T cells, B cells, macrophages, and dendritic cells.⁷ PD-L1 plays a pivotal role in mediating the immune response against tumor cells through binding to the immune-inhibitory receptor known as programmed cell death protein 1 (PD-1), a member of the B7-CD28 gene superfamily.⁸ PD-1 on immune cells binds to PD-L1 expressed on the surface of tumor cells, resulting in the inhibition of T-cell activation through the T-cell receptor and CD28, and the attenuation of T-cell-mediated anti-cancer immunity.⁹ More importantly, PD-L1 overexpression has been reported to be associated with enhanced neoplastic growth, chemotherapy resistance, and cancer recurrence.^{10,11} Accordingly, several immunotherapeutic modalities have been developed for the treatment of different cancer types, including breast cancer, to disrupt the interaction of PD-L1 with the PD-1 receptor.^{12–15} Consequently, the Food and Drug Administration (FDA) approved the PD-L1 inhibitor atezolizumab and the PD-1 inhibitor pembrolizumab in combination with chemotherapy for the treatment of patients diagnosed with locally advanced or metastatic PD-L1-positive TNBC,¹⁶ suggesting that immunotherapy is now becoming a major component of breast cancer treatment.

Recent evidence has demonstrated that NAC can induce the upregulation of immunosuppressive molecules, such as PD-L1, in many solid tumors including cervical cancer, non-small cell lung cancer, ovarian cancer, head

and neck squamous cell carcinoma, squamous oral carcinoma, as well as breast cancer.^{17–21} In breast cancer, a significant increase in the expression of PD-L1 in patients who received NAC in combination with trastuzumab prior to surgery has been reported.²¹ On the contrary, other reports suggested that breast cancer patients receiving NAC developed a notable reduction in the expression rate of PD-L1,^{22,23} indicating that the exact alterations in PD-L1 expression upon exposure of tumor cells to NAC are still unclear.

It is known that the interaction between the immune system and tumor cells within the tumor microenvironment contributes, in part, to the control of tumor progression.²⁴ Tumor-infiltrating lymphocytes (TILs) play a critical role in the host's anti-tumor immune response across different types of malignant tumors.^{25,26} Importantly, a higher PD-L1 expression in breast cancer is closely associated with the increased extent of TILs.^{27–29} The effect of TILs on clinical outcome has been extensively investigated in different breast cancer subtypes,^{30,31} in that, a higher count of TILs in the core-needle biopsies prior to NAC is likely to be associated with a complete PR following exposure to NAC.^{32–36} Nevertheless, other reports did not establish the potential for the extent of TILs prior to NAC to predict the PR in breast cancer.^{30,37,38} Accordingly, the association between TILs, PD-L1 expression and response to NAC in breast cancer is not yet established, especially in patients' tumors that develop partial or incomplete PR to NAC.

In the current study, we investigated PD-L1 immunorexpression based on three scoring approaches in invasive ductal carcinoma of the breast (IDC) of patients with incomplete PR to NAC and its associations with short-term therapy outcomes. Moreover, we examined the extent of TILs pre- and post-NAC and its association with PD-L1 status and clinicopathological characteristics of the patients.

Patients and methods

Patient specimens

A total number of 60 patients ($n = 60$) that have been diagnosed with IDC and who were treated with NAC (prior to surgical resection) were included in the study. In addition, 31 pre-operative core-needle biopsy samples matched to the surgically resected samples were collected to compare PD-L1 expression status pre- and post-NAC ($n = 31$). The formalin-fixed paraffin-embedded (FFPE) tissue specimens were obtained from the Pathology Department archives of Jordan Royal Medical Services (JRMS) and Prince Hamza Hospital (PHH), Amman, Jordan, between the years 2017 and 2021. Clinicopathological data and types of NAC were retrieved from the Departments of Surgery and Pathology in both institutions. This retrospective study was approved by the Internal Review Board

committees at both JRMS (ID No. 9/2019) and PHH (ID No. 32/2778). All patients signed a written informed consent form according to the recommendations of the Declaration of Helsinki.

The inclusion criteria were: (1) IDC patients of stage I-III who received full cycles of NAC prior to surgery; (2) patients who had poor PR following NAC and (3) patients who underwent wide local excision or modified radical mastectomy following NAC, while the exclusion criteria were: (1) patients who also received neoadjuvant hormone therapy and/or neoadjuvant radiotherapy in addition to NAC; (2) patients who did not receive NAC prior to surgery or who did not fully complete cycles of NAC; (3) patients who received a type of anti-PD-1/PD-L1 therapy; (4) patients with any form of established metastatic disease (stage IV); (5) patients who did not undergo surgical resection following the completion of NAC and (6) patients whose FFPE tissue blocks were not available.

Immunohistochemical procedures

The formalin-fixed paraffin-embedded tissue blocks were cut using a microtome (Leica, RM2125RT, Germany) into 4- μ m-thick sections and placed on positive charged glass slides. The tissue sections were dewaxed using two containers of fresh xylene solution for 5 min and then rehydrated in graded concentrations of ethanol (100%, 95%, and 70%). Antigen unmasking was performed using Envision Flex target retrieval solution (Agilent, Dako, USA), at a low pH of 6.0 for 20 min at 95°C in a water-bath and then cooled for 30 min at room temperature as described previously.³⁹ To block nonspecific endogenous peroxidase activity, the tissue sections were treated with a peroxidase-blocking reagent for 10 min at room temperature. Nonspecific reactions were blocked by animal serum blocking reagent G (CTS002; R&D Systems, Inc., Minneapolis, MN, USA) for 20 min at room temperature. Subsequently, tissue sections were incubated with monoclonal mouse anti-PD-L1 (3 μ g/mL, Clone 22C3, ref: SK006, Dako, USA), for 2 h at room temperature. Next, tissue samples were incubated with secondary Envision FLEX mouse linker antibody for 15 min at room temperature. Subsequently, tissue samples were incubated with Envision FLEX HRP-labeled polymer reagent for 25 min at room temperature. The antigen-antibody complex was visualized by application of chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako, USA) for 10 min at room temperature followed by DAB enhancer reagent (cupric sulfate in water, PD-L1 IHC 22C3 pharmDx, SK006, Agilent Dako) for 10 min at room temperature.

After each incubation step, except for the blocking step with serum, tissue sections were rinsed with phosphate-buffered saline (PBS) solution (Bio-Rad

Laboratories, ref: 31,098, Inc. France) with 0.1% Tween 20 detergent (Sigma, 67f-0826, USA). Slides were then counterstained with hematoxylin for 5 min and then rinsed with tap water. Next, slides were dehydrated in an ethanol series (70%, 95%, and 100%), and then rinsed in xylene. Finally, slides were mounted with dibutyl phthalate in xylene mounting media (#BCBX0183, Sigma, Germany) and coverslipped. Positive and negative controls were performed along with each staining series. The positive control included human placenta and human tonsil tissue samples, while the negative control was performed by the omission of primary antibody and substitution with PBS.

Evaluation of tumor-infiltrating lymphocytes

The count of stromal TILs for all cases was evaluated on a representative slide stained with hematoxylin and eosin (H&E) by a pathologist who was blinded to all clinicopathological information of the patients and according to the International TILs Working Group on Breast Cancer (2014).⁴⁰ Stromal TILs were evaluated as the average percentage of mononuclear immune cells in the tumor stromal area. Areas of central necrosis or fibrosis were excluded from the evaluation. According to International TILs Working Group guidelines, the cases were classified into three groups, namely, low-TIL group (0–10%), intermediate-TIL group (11–40%) and high-TIL group (41–100%).⁴⁰

Evaluation of PD-L1 immunostaining

PD-L1 immunoexpression in breast cancer was assessed by a pathologist according to different scoring approaches, including the tumor proportion score (TPS), immune cell score (ICS), and combined tumor and immune cell score (combined positive score, CPS). PD-L1 protein expression in tumor cells (TCs) was calculated based on the percentage of invasive carcinoma cells showing partial or complete membranous staining pattern regardless of the staining intensity.⁴¹ ICS was calculated as the number of the immune cells (ICs) with membranous and/or cytoplasmic staining patterns divided by the total number of viable tumor cells.⁴² CPS was calculated as the number of PD-L1 positive cells on both TCs and ICs divided by the total number of viable tumor cells, multiplied by 100.⁴³ In the present study, we assessed the PD-L1 immunoexpression using all three scoring approaches with a 1% cut-off according to previous studies.^{44–46} PD-L1 stained slides were visualized using a light microscope (Olympus CX 41, Olympus, Tokyo, Japan) under 200x, 400x magnification. Images were captured at 600x magnification on a digital camera attached to Olympus BX 53 microscope.

Table I. Clinicopathological characteristics of study population.

Characteristic	Number	%	Characteristic	Number	%
Age (Years)			ER		
<50	30	50.0	Positive	49	81.7
≥50	30	50.0	Negative	11	18.3
Histologic grade			PgR		
Grade-I	2	3.3	Positive	48	80.0
Grade-II	31	51.7	Negative	12	20.0
Grade-III	27	45.0	HER-2 status		
Tumor stage/tumor size			Positive	12	20.0
T1	11	18.3	Negative	48	80.0
T2	25	41.7	Recurrence		
T3/T4	24	40.0	Yes	8	13.3
Molecular subtypes			No	52	86.7
Luminal A	42	70.0			
Luminal B	9	15.0			
HER-2-enriched	2	3.3			
TNBC	7	11.7			
Nodal status					
Positive	41	68.3			
Negative	19	31.7			
Lymphovascular invasion					
Present	40	66.7			
Absent	20	33.3			

Note: Table shows the main clinicopathological data of patients including age, tumor stage, tumor grade, nodal status, lymphovascular invasion status, hormone receptor expression status including ER: Estrogen Receptor, PgR: Progesterone Receptor and HER-2: Human Epidermal Growth Factor Receptor 2, and lastly, cancer recurrence. All patients underwent wide local excision or modified radical mastectomy following NAC administration. Among those, 55 (91.7%) received the docetaxel, TAC: Adriamycin and cyclophosphamide regimen, while 5 (8.3%) received the CEF: cyclophosphamide, epirubicin, 5-fluorouracil regimen. Patients with HER-2-positive breast cancer received trastuzumab adjuvant therapy. The median age of the patients was 49.5 (range: 28–71) years. The median follow-up period was 25 months.

Statistical analysis

The sample size was calculated using Power Analysis and Sample Size Program 2021 (PASS, NCSS Statistical Software, Kaysville, UT, USA). Based on the PASS (Alpha at 0.05 and Power at 0.8), a minimum sample size of 60 patients was required for the current analysis based on the followed inclusion and exclusion criteria and a previous study by Pelekanou et al.²² Fisher's exact test was used to determine the correlation between the expression of PD-L1 and TILs with clinicopathological variables such as tumor grade, tumor stage/size, nodal status, lymphovascular invasion, different molecular subtypes (based on receptor expression), and cancer recurrence. PD-L1 expression status pre- and post-NAC was compared using the McNemar's test. Kaplan–Meier's method with Log-rank test was used to measure the impact of PD-L1 immunorexpression on the overall survival rate. Overall survival was calculated from the date of completion of NAC to the date of death or last documented follow-up. Comparisons between pre- and post NAC levels of TILs were performed by the unpaired Student's t-test and Wilcoxon's

signed-rank test. The relationship between different scoring approaches of PD-L1 (TPS, IC, and CPS) was established using Pearson's correlation coefficient method. Statistical data analyses were performed using Statistical Package for the Social Sciences (SPSS) version 28 (SPSS Inc, Chicago, IL, US). Kaplan–Meier curves were performed with Graphpad Prism version 9.2.0 (GraphPad Software, San Diego, California, USA). A *p* value of 0.05 or lower was regarded as statistically significant.

Results

Clinicopathological characteristics of patients' samples

In order to examine the expression of PD-L1, we collected 60 tumor samples from patients with an established diagnosis of IDC of the breast who have received a type of NAC, including 31 matched core-needle biopsies of breast tumor tissue prior to receiving NAC. The median age of the patients was 49.5 years (range: 28–71). All patients received a

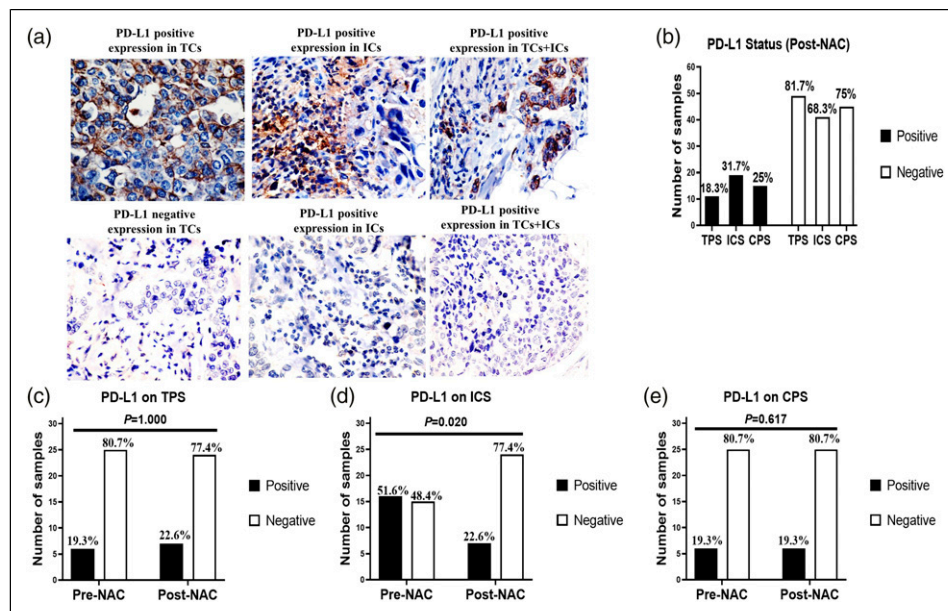


Figure 1. PD-L1 positive expression rates in tumor cells and infiltrating immune cells pre- and post-NAC in breast cancer samples. (a). Representative images of positive (greater than 1% of PD-L1 staining in TCs by TPS, ICs by ICS, and TCs+ICs by CPS) or negative PD-L1 expression in breast cancer tissue post-NAC (Original magnification 600x). (b). Graph represents PD-L1 immunoreexpression levels post-NAC in the total number of surgically resected tumor samples ($n = 60$) based on three scoring approaches TPS, ICS and CPS. PD-L1 was more frequently expressed based on ICS than by TPS or CPS (31.7% vs. 18.3% or 25%; respectively). (c, d and e) Comparison of the PD-L1 positive expression rate (number of cases and percentages per sample) between pre- and post-NAC samples based on TPS, ICS and CPS in 31 matched breast cancer tumors ($n = 31$). p values were calculated using Wilcoxon's Signed-Rank test. *Abbreviations:* TCs: tumor cells; ICs: immune cells; TPS: tumor proportion score; ICS: immune cell score; CPS: combined positive score; NAC: neoadjuvant chemotherapy.

NAC regimen prior to surgical excision of the tumor. Among these, 55 (91.7%) patients received the docetaxel, Adriamycin and cyclophosphamide (TAC) regimen, while remaining 5 patients (8.3%) received the cyclophosphamide, epirubicin, 5-fluorouracil (CEF) regimen. Only patients with HER-2-positive breast cancer received trastuzumab adjuvant therapy. Clinicopathologic characteristics of the patients with IDC of the breast are summarized in Table 1. All patients were diagnosed with primary non-metastatic IDC breast carcinoma of stage I–III and developed poor or incomplete PR to NAC. According to the American Joint Committee on Cancer (AJCC's) seventh Edition of Cancer Staging Manual,⁴⁷ 11 (18.3%) of the pathologically staged tumors were pT1, 25 (41.7%) were pT2 and 24 (40%) were pT3/T4. Molecular subtypes of breast cancer were classified based on the immunoreexpression status of ER, PgR, and HER-2 as follows: luminal A phenotype (ER or/and PgR positive and HER-2 negative); luminal B phenotype (ER or/and PgR positive and HER-2 positive), HER-2-enriched, and TNBC subtype (ER, PgR, and HER-2 negative).⁴⁸ The distribution of tumor molecular subtypes in the sample was as follows: 42 (70%) tumors were luminal A, 9 (15%) tumors were luminal B, 11

(18.3%) tumors were HER-2-enriched, and 7 (11.7%) tumors were TNBC (Table 1).

PD-L1 positive expression rates in tumor cells and infiltrating immune cells pre- and post-NAC in IDC breast samples

Next, we wanted to directly determine the expression level of PD-L1 in all the samples utilizing TPS, ICS, or CPS. Immunodetection of PD-L1 was performed in all 60 surgically resected tumor samples following NAC, and in the 31 corresponding baseline core-needle biopsy samples prior to receiving NAC (Figure 1(a)). The remaining core specimens were unavailable in the Pathology Department. First, among the post-NAC 60 cases investigated, positive PD-L1 immunostaining was observed in 11 (18.3%) of cases by TPS, 19 (31.7%) by ICS, and 15 (25%) by CPS (Figure 1(b)). Positive expression rates of PD-L1 in luminal A tumors were as follows: 7 (16.7%) tumors by TPS, 11 (26.2%) tumors by ICS, and 9 (21.4%) tumors by CPS (Table 2). In the luminal B tumors, positive expression rates of PD-L1 were: 1 (11.1%) tumor by TPS, 4 (44.4%) tumors by ICS and 2 (22.2%) tumors by CPS (Table 2). In the HER-2-enriched cases, positive expression rates of PD-L1

Table 2. Association of PD-L1 expression using three scoring approaches and TILs count with clinicopathological variables.

Variables	PD-L1 in TCs			PD-L1 by CPS			PD-L1 in ICs			TILs Count		
	Positive	Negative	p	Positive	Negative	p	Positive	Negative	p	Low	Intermediate	p
	n (%)	n (%)		n (%)	n (%)		n (%)	n (%)		n (%)	n (%)	
Histologic grade												
Grade-I	0 (0)	2 (100)	0.790	0 (0)	2 (100)	0.868	0 (0)	2 (100)	0.210	2 (100)	0 (0)	1.000
Grade-II	6 (19.3)	25 (80.7)		9 (29)	22 (71)		13 (41.9)	18 (58.1)		28 (90.3)	3 (9.7)	
Grade-III	5 (18.5)	22 (81.5)		6 (22.2)	21 (77.8)		6 (22.2)	21 (77.8)		24 (88.9)	3 (11.1)	
Tumor stage/tumor size												
T1	2 (18.2)	9 (81.8)	0.501	2 (18.2)	9 (81.2)	0.822	1 (9.1)	10 (90.9)	0.180	10 (90.9)	1 (9.1)	0.473
T2	3 (12)	22 (88)		7 (28)	18 (72)		10 (40)	15 (60)		21 (84)	4 (16)	
T3/T4	6 (25)	18 (75)		6 (25)	18 (75)		8 (33.3)	16 (66.7)		23 (95.8)	1 (4.2)	
Nodal status												
Positive	7 (17.1)	34 (82.9)	0.730	10 (24.4)	31 (75.6)	1.000	13 (31.7)	28 (68.3)	1.000	38 (87)	3 (13)	0.370
Negative	4 (21.1)	15 (78.9)		5 (26.3)	14 (73.7)		6 (31.6)	13 (68.4)		16 (43)	3 (57)	
Lymphovascular invasion												
Present	7 (17.5)	33 (82.5)	1.000	10 (25)	30 (75)	1.000	12 (30)	28 (70)	0.772	36 (90)	4 (10)	1.000
Absent	4 (20)	16 (80)		5 (25)	15 (75)		7 (35)	13 (65)		18 (90)	2 (10)	
ER status												
Positive	6 (12.2)	43 (87.8)	0.022	9 (18.4)	40 (81.6)	0.021	13 (26.5)	36 (73.5)	0.086	46 (93.9)	3 (6.1)	0.069
Negative	5 (45.5)	6 (54.5)		6 (54.5)	5 (45.5)		6 (54.5)	5 (45.5)		8 (72.7)	3 (27.3)	
PgR status												
Positive	8 (16.7)	40 (83.3)	0.677	11 (22.9)	37 (77.1)	0.472	15 (31.2)	33 (68.8)	1.000	44 (91.7)	4 (8.3)	0.590
Negative	3 (25)	9 (75)		4 (33.3)	8 (66.7)		4 (33.3)	8 (66.7)		10 (83.3)	2 (16.7)	
HER-2 status												
Positive	1 (9.1)	10 (90.9)	0.670	3 (27.3)	8 (72.7)	1.000	5 (45.5)	6 (54.5)	0.301	9 (81.8)	2 (18.2)	0.302
Negative	10 (20.4)	39 (79.6)		12 (24.5)	37 (75.5)		14 (28.6)	35 (71.4)		45 (91.8)	4 (8.2)	
Molecular subtypes												
Luminal A	7 (16.7)	35 (83.3)	0.349	9 (21.4)	33 (78.6)	0.389	11 (26.2)	31 (73.8)	0.468	40 (95.2)	2 (4.8)	0.084
Luminal B	1 (11.1)	8 (88.9)		2 (22.2)	7 (77.8)		4 (44.4)	5 (55.6)		7 (77.8)	2 (22.2)	
HER2-enriched	0 (0.0)	2 (100.0)		1 (50.0)	1 (50.0)		1 (50.0)	1 (50.0)		2 (100.0)	0 (0.0)	
TNBC	3 (42.9)	4 (57.1)		3 (42.9)	4 (57.1)		3 (42.9)	4 (57.1)		5 (71.4)	2 (28.6)	
Recurrence												
Yes	1 (12.5)	7 (87.5)	1.000	4 (50)	4 (50)	0.098	4 (50)	4 (50)	0.249	8 (100)	0 (0)	0.585
No	10 (19.2)	42 (80.8)		11 (21.2)	41 (78.8)		15 (28.8)	37 (71.2)		46 (88.5)	6 (11.5)	

Table shows no statistical association between PD-L1 protein expression in TCs, CPS, and ICs with clinicopathological variables except for a significant association with ER status in TCs or CPS ($p = 0.022$, $p = 0.021$; respectively). No statistical association was found between TILs and clinicopathological variables. p -values were determined using a Fisher's exact test.
 Note: TCs: tumor cells, ICs: immune cells, CPS: combined positive score, TILs: tumor infiltrating lymphocytes, ER: estrogen receptor, PgR: progesterone receptor, HER-2: human epidermal growth factor receptor 2, TNBC: triple-negative breast cancer.

were as follows: 0 (0.0%) tumor by TPS, 1 (50.0%) tumors by ICS, and 1 (50.0%) by CPS (Table 2). Lastly, PD-L1 expression was observed in 3 (42.9%) of TNBC samples utilizing the three scoring approaches (Table 2). We found that the molecular subtype of breast cancer is not typically associated with PD-L1 expression status according to the TPS, CPS, or ICS approaches ($p = 0.349$, $p = 0.389$, and $p = 0.468$; respectively) (Table 2). However, PD-L1 immunoexpression rates were higher in TNBC according to TPS and CPS in comparison with other molecular breast subtypes, but was not statistically significant. Moreover, we observed that PD-L1 immunoexpression levels based on ICS were lower, although not significantly, in luminal A samples compared to the other breast cancer subtypes. In total, the positivity rate of PD-L1 in ICs post-NAC resection samples was 31.7% (19/60) based on ICS, while it was 18.3% (11/60) in tumor cells (TCs) based on TPS, indicating that PD-L1 expression level following NAC was higher in ICs compared to TCs (31.7% vs. 18.3%) (Figure 1(b)).

We, then, compared PD-L1 expression rates in the 31 matched samples pre- and post-exposure to NAC. PD-L1 positive expression rates in the pre-NAC samples were as follows: 6 (19.3%) of samples by TPS, 16 (51.6%) by ICS, and 6 (19.3%) by CPS, while in comparison, PD-L1 positive expression rates in the matched post-NAC samples were as follows: 7 (22.6%) of samples by TPS, 7 (22.6%) by ICS, and 6 (19.3%) by CPS in (Figure 1(c), 1(d) and 1(e)). Accordingly, PD-L1 expression was significantly decreased in ICs based on ICS in breast tumor samples following exposure to NAC (McNemar's: $p = 0.020$), while a significant difference in PD-L1 positive expression in TCs based on TPS or CPS was not identified (McNemar's: $p = 1.000$, $p = 0.617$, respectively). (Figure 1(c), 1(d) and 1(e)).

Finally, The PD-L1 concordance rate among the three scoring approaches reached 75% (45/60) of cases, including 13.3% (8/60) which had positive expression and 61.7% (37/60) with negative expression for PD-L1. We found that PD-L1 expression in TCs was moderately positively correlated with PD-L1 expression based on CPS and weaker with PD-L1 expression in ICs (Pearson's correlation coefficient = 0.522 and 0.418, $p = 0.00002$ and $p = 0.001$, respectively). On the other hand, there was a strong positive correlation between PD-L1 expression based on CPS and PD-L1 expression in ICs (Pearson's correlation coefficient = 0.848, $p < 0.00001$).

Correlation of PD-L1 expression with clinicopathologic characteristics

After determining the positive expression rates of PD-L1 in tumor and infiltrating immune cells, we wanted to determine whether there was a correlation between the three

utilized expression scores and several clinicopathological characteristics of the patients' sample. Our analysis identified that there was only a statistically significant association between PD-L1 positive expression based on TPS and CPS with ER status ($p = 0.022$, $p = 0.021$; respectively) (Table 2). PD-L1 positive expression based on the three different scoring approaches (TPS, ICS and CPS) showed a non-significant association with HER-2 and PgR status ($p > 0.05$) (Table 2). There was no statistically significant association between PD-L1 positive expression and lymphovascular invasion or lymph nodal status ($p > 0.05$). In addition, PD-L1 positive expression showed no association with grade and stage of tumors ($p > 0.05$). Lastly, PD-L1 positive expression was not associated with the extent of cancer recurrence ($p > 0.05$) (Table 2). It is noteworthy that based to our inclusion criteria, only tumors staged I-III were included in the analysis and monitored for relapse, while the expression of PD-L1 was not evaluated in patients with established metastasis.

Tumor-infiltrating lymphocytes post-NAC and its association with PD-L1 status

Next, we wanted to determine the extent of stromal TILs in breast tumor samples. TILs were assessed in all 60 post-NAC samples including the 31 matched pre-NAC samples (Figure 2(a)). The highest proportion of stromal TILs in breast cancer samples was 40% (no high-level TILs count was identified in any of the samples). The median stromal TILs count in the total 60 post-NAC samples was 5% (range 0–40%) (Figure 2(b)). The median TILs count in 31 matched pre-NAC and post-NAC samples was 5% (range 0–20%) and 1% (range 0–30%), respectively (Figure 2(c)). However, we observed no statistically significant difference in the count of TILs in the 31 matched pre- and post-NAC samples ($t = 0.581$, $p = 0.563$ or Wilcoxon Signed Rank test; $z = -0.625$, $p = 0.529$) (Figure 2(c)), indicating that exposure to NAC did not have a significant effect on the extent of lymphocytes infiltration. Moreover, TILs level in the total 60 post-NAC samples was not significantly correlated with any of clinicopathologic variables in our analysis (Table 2). Finally, there was no significant association between PD-L1 immunoexpression based on the three scoring approaches (TPS, ICS, and CPS) with the extent of TILs (Table 3).

Kaplan-Meier survival analysis

Lastly, we evaluated the impact of PD-L1 immunoexpression on overall survival. The follow-up duration of patients ranged from 6 months to 58 months (median follow-up, 25 months). Of the total 60 patients, 8 (13.3%) of cases developed confirmed cancer relapse using clinical and

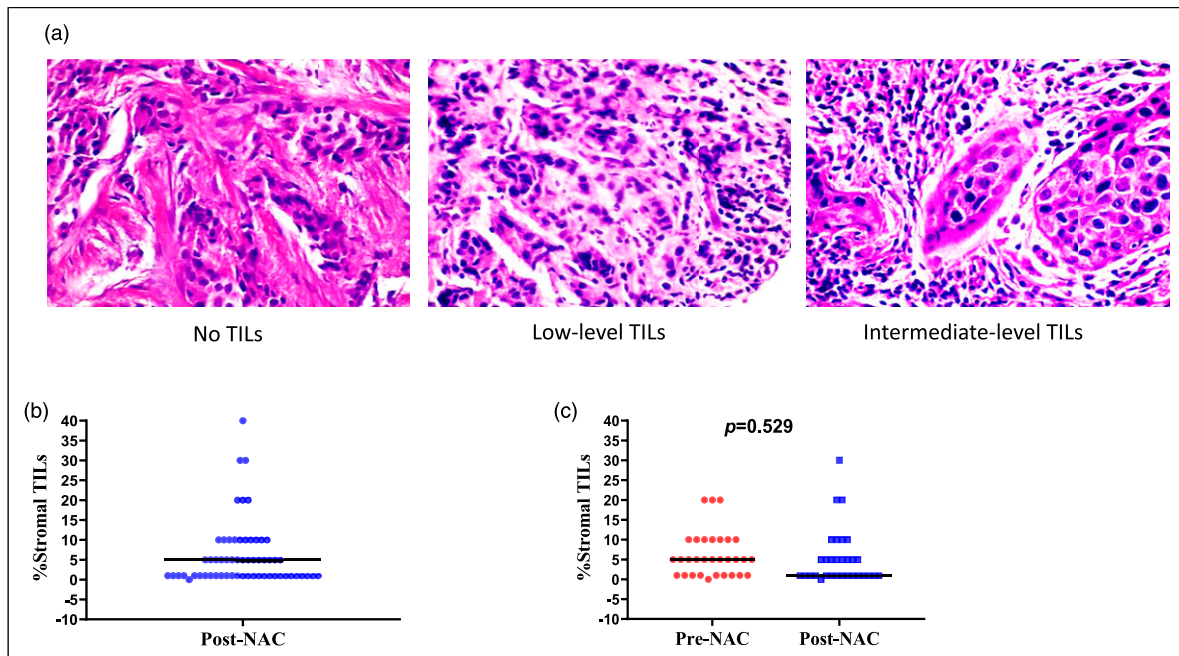


Figure 2. Tumor-infiltrating lymphocytes count post-NAC in breast cancer samples. (a). Representative images of TILs levels based on H&E staining. TILs count was categorized as no TILs (0%), low (0–10%), and intermediated (11–40%) based on International TILs Working Group on Breast Cancer (2014). (Original magnification 600x). (b) A strip plot showing the distribution of TILs count levels in the post-NAC samples with a median of 5%. (c) A strip plot showing no significant difference in the TILs count pre- and post-NAC. p value was calculated using Wilcoxon's Signed-Rank test.

Table 3. Relationship between PD-L1 immunopositivity using different scoring approaches (TPS, CPS, and ICS) and TIL status.

	PD-L1 on TCs			PD-L1 on CPS			PD-L1 on ICs		
	Positive n (%)	Negative n (%)	p	Positive n (%)	Negative n (%)	p	Positive n (%)	Negative n (%)	p
TILs									
Low levels ($n = 54$)	10 (90.9)	44 (89.8)	1.000	12 (80)	42 (93.3)	0.159	16 (84.2)	38 (92.7)	0.370
Intermediate levels ($n = 6$)	1 (9.1)	5 (10.2)		3 (20)	3 (6.7)		3 (15.8)	3 (7.3)	

Note: Table shows no statistically significant association between PD-L1 immunopositivity by different scoring approaches and TILs. p values were calculated using a Fisher's exact test.

radiological assessment, while only 9 (15%) patients were deceased (Table 2). Kaplan–Meier analysis indicated that there was no statistically significant association between PD-L1 immunopositivity based on TPS, ICS, and CPS with overall survival (Log-Rank test, $p = 0.051$, $p = 0.235$, $p = 0.472$; respectively) (Figure 3). However, we observed that positive PD-L1 immunostaining in TCs showed a trend of worse overall survival despite not achieving statistical significance ($p = 0.051$) (Figure 3).

Discussion

In addition to the currently utilized approaches for breast cancer treatment, the US FDA approved PD-L1 immune checkpoint inhibitors for late-stage invasive breast cancer patients.⁴⁹ Therefore, a subpopulation of breast cancer patients will be receiving NAC, a standard treatment for breast cancer, in combination with immune checkpoint inhibitors, which invites for the investigation of how the exposure to NAC will potentially affect the response to

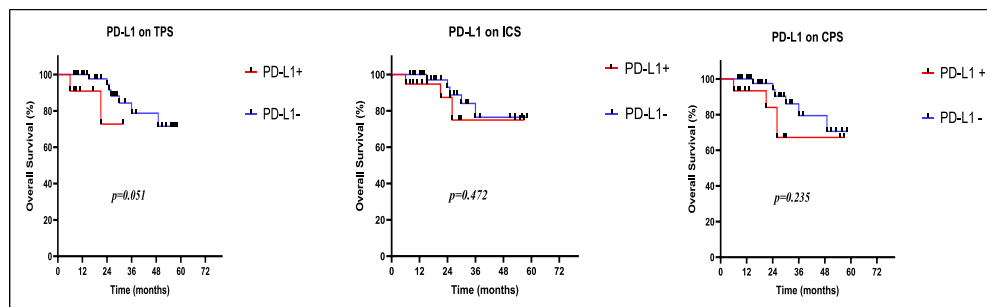


Figure 3. Survival rates for breast cancer patients according to the positive expression rates of PD-L1 by three scoring approaches. (a), (b) and (c). Kaplan-Meier curves showing no significant association between PD-L1 status using the three scoring approaches and overall survival. A strong trend between patients with PD-L1 expression in TCs with overall survival was recorded although did not reach statistical significance ($p = 0.051$) as shown in A. p values were calculated using the log-rank test.

immunotherapy. In this study, we provide an analysis of the PD-L1 expression levels on tumor and immune cells of breast cancer patients treated with NAC with focus on those who developed poor or no PR.

Several studies have previously investigated the expression of the PD-L1 in breast cancer subtypes prior to the exposure to cancer therapy (ranged from 0.5% to 24% of tumor cells).^{50–55} For example, a meta-analysis of 38 reports of 4184 breast cancer samples revealed that the overall expression rate of PD-L1 in breast tumor tissue was 24% in TCs, 33% in ICs, and 25% according to CPS.⁵³ In addition, Guo et al. demonstrated that the expression rate of PD-L1 in breast cancer patients who did not receive NAC was 10% in TCs, 16% in ICs, and 20% according to CPS.⁴⁴ In the present study, we found that the positive expression rate of PD-L1 in core-needle biopsy sample prior to NAC exposure was 19.3% in TCs based on TPS, 51.6% in ICs based on ICS, and 19.3% based on CPS.

While the increased expression of PD-L1 in breast TCs in response to chemotherapeutic agents *in vitro* has been established,^{56,57} few studies evaluated the role of NAC in altering the expression levels of PD-L1 in breast tumor tissue.^{21–23,58} Due to the existence of conflicting observations on alterations in the expression levels of PD-L1 following exposure to NAC in breast cancer samples, we sought to provide a delineation of the main findings available in the literature (Table 4). Previously studied breast cancer subtypes for PD-L1 expression following NAC included triple-negative or hormone receptor-positive (6 studies), while the number of studies where breast cancer histopathological subtyping was not provided was 4. The sample size across studies ranged from 30 to 309 patients (Table 4). The investigated NAC regimens included CEF, cyclophosphamide, Adriamycin and 5-Fluorouracil (CAF), epirubicin/cyclophosphamide (EC) and TAC with or without neoadjuvant hormonal therapy. The positive expression rate of PD-L1 following NAC using different scoring approaches ranged from 13.3% to 87.3% with a mean of 31.5%

(Table 4). It is noteworthy to mention that these percentages are inclusive of both cytoplasmic and membranous expression of PD-L1. The number of studies that indicated a decrease in PD-L1 expression following NAC (cytoplasmic or membranous) was 7, while the number of studies that showed an increase in PD-L1 expression following NAC (cytoplasmic or membranous) was 2 (Table 4).

Collectively, this previous literature indicates that PD-L1 expression in TCs is altered following NAC exposure, but also reflects variability in the expression status of PD-L1 in breast tumor samples exposed to NAC. For example, Srivastava et al. reported that the PD-L1 immunoexpression rate in TCs using H-score prior to NAC exposure in locally advanced breast cancer was 36.7% (11/30), whereas PD-L1 immunoexpression rate following NAC was 13.3% (4/30).²³ Similarly, Pelekanou et al. investigated PD-L1 immunoexpression rate in matched pre- and post-NAC residual invasive breast cancer and showed that 17.2% (10/58) of cases were PD-L1 positive in tumor and/or stroma post-NAC while 51.02% (21/41) of cases were PD-L1-positive in pre-NAC.²² On the other hand, Chen et al. investigated PD-L1 immunoexpression in residual invasive breast carcinoma after receiving different types of NAC regimens, and remarkably, the cytoplasmic, rather than membranous, immunoexpression rate of PD-L1 in tumor cells was positive in 49.5% of samples.⁵⁹ Grandal et al. showed that PD-L1 immunoexpression level in patients with residual TNBC following receiving a neoadjuvant anthracycline-based regimen was 31/89 (34.8%) in TCs and 30/89 (33.7%) in ICs.⁶⁰ In this regard, Asano et al. also revealed that the immunoexpression level of PD-L1 in TCs in triple-negative and HER2-positive breast cancer patients ($n = 177$) following NAC was 23.7%.⁶¹ Guo et al. showed that the expression rate of PD-L1 in breast cancer patients who received NAC was 8.5% in TCs, 14.5% in ICs, and 18.3% based on CPS. While these studies did not perform a comparison with pre-NAC PD-L1 expression levels, they still reflect relatively high PD-L1

Table 4. An overview of several previous studies investigating alterations in PD-L1 expression following NAC in invasive breast cancer.

Molecular subtype	NAC	Sample size	Approach	Antibody (clone, supplier)	Expression pattern	Cut-off	Pre NAC PD-LI positivity	Post NAC PD-LI positivity	Median follow-up (months)	Reference
No specific type	AC*4 + T*4 (56%)	58 (including 41 paired samples)	QIF	monoclonal antibody EIL3N clone (Cell Signaling Technology)	N/A	500 AU (tumor and/or stroma)	51.0% (21/41)	17.2% (10/58)	60	22
No specific type	CAF + T (76.7%)	30	IHC	polyclonal antibody (Abcam)	Membrane	H-scores PD-LI ≥ 100 = negative/low, 100-300 = high/positive	11 (36.7%)	4 (13.3%)	N/A	23
HER-2 positive	A+T and trastuzumab	126	IHC	SP142 clone (Spring Bioscience, USA)	Membrane/ Cytoplasm	Tumor proportion score $\geq 1\%$	N/A	22 (17.5%)	52.5	21
No specific type	CEF (16.2%) or NE (38.8%) or PC (45.0%)	309	IHC	polyclonal antibody (ab205921 Abcam)	Cytoplasm	The median density (low: 0.001-0.022, high: >0.022)	N/A	153 (PD-LI high density), 156 (PD-LI low density)	70	59
All breast cancers	CEF*4 + T*12	177	IHC	clone 27A2 (MBL, Japan)	Membrane/ Cytoplasm	$\geq 10\%$ (high expression)	N/A	42 (23.7%)	40	61
Hormone receptor-positive/ HER2-negative	B followed by AC + hormone therapy	96	IHC	clone 9A11 (Cell Signaling)	Membrane/ Cytoplasm	Tumor proportion score $\geq 1\%$	N/A	22%	94.8	73
TNBC	AC or EC or A+T	50 (including 34 paired samples)	IHC	monoclonal antibody, SP263 clone (Ventana Medical Systems)	Membrane	PD-LI H-score ≥ 5	58.8% (20/34)	56.7% (17/30) 46.7% (14/30)	N/A	71

(continued)

Table 4. (continued)

Molecular subtype	NAC	Sample size	Approach	Antibody (clone, supplier)	Expression pattern	Cut-off	Pre NAC PD-LI positivity	Post NAC PD-LI positivity	Median follow-up (months)	Reference
TNBC	Anthracycline or sequential anthracycline-taxane regimens	89	IHC	E1L3N clone (Cell Signaling)	Membrane	Tumor proportion score $\geq 1\%$	N/A	31 (34.8%)	80	60
No specific type	B + nab-paclitaxel, followed by AC	202 (120 pre-NAC, 43 post-NAC and 39 paired samples)	IHC	clone 22C3 (Dako)	Membrane	Tumor proportion score $\geq 1\%$	16/39 (41%)	13/39 (33.3%) 14/43 (33%)	N/A	58
TNBC	durvalumab or placebo + nab-paclitaxel, followed by EC	174	IHC	clone SP263 (Ventana Medical Systems)	Membrane	Tumor proportion score $\geq 1\%$	N/A	Tumor and/or immune cells 138/158 (87.3%)	N/A	64

Table outlines the conflicting findings in the literature of PD-L1 immunoeexpression post-NAC in different types of breast cancer. The parameters of focus include types of neoadjuvant chemotherapy (NAC) regimens used in each study, sample size, immunodetection methods, types of primary antibody clones used, immunoeexpression pattern (membranous vs cytoplasmic), immunoeexpression rate pre- and post- NAC, type of scoring system, and lastly, the median patient follow-up period. Asterisks indicate number of chemotherapy cycles. The % symbol in the NAC column represents the proportion of patients who received NAC.

Note: RIBC: residual invasive breast carcinoma, TNBC: triple-negative breast cancer, B: bevacizumab, LABC: locally advanced breast cancer, N/A: not available, QIF: quantitative immunofluorescence, IHC: immunohistochemistry, AC: adriamycin plus cyclophosphamide, T: docetaxel, CAF: cyclophosphamide, Adriamycin and 5-fluorouracil, CEF: cyclophosphamide, epirubicin, 5-fluorouracil, NE: navelbine and epirubicin, PC: paclitaxel-carboplatin, EC: epirubicin/cyclophosphamide.

expression levels in post-NAC samples. In our study, the expression rate of PD-L1 in IDC specimens was 22.6% of cases in TCs based on TPS, 22.6% in ICs based on ICS, and 19.3% according to CPS in matched post-NAC samples. More specifically, in the 31 matched pre- and post-NAC samples, 22 samples showed invariant results (i.e. remained negative following NAC).

Several studies demonstrated that PD-L1 immunorepression rates were high in the TNBC subtype compared to other breast cancer subtypes.^{54,62–64} Moreover, a previous study indicated that PD-L1 was overexpressed in TNBC and HER-2-positive breast cancer.⁶⁵ The prevalence of PD-L1 positivity was observed more than twice as much in patients with TNBC than in luminal B/HER-2-negative breast cancer.⁶⁶ Paradoxically, a previous study showed that the PD-L1 expression rate was higher in the luminal A subtypes compared to other breast cancer subtypes including TNBC.⁶⁷ In post-NAC specimens collected through surgical resection, we found that PD-L1 immunorepression levels according to TPS and CPS were high in the TNBC subtype compared to other breast cancer subtypes, albeit not statistically significant. Furthermore, we observed that PD-L1 immunorepression levels based on ICs were specifically lower in luminal A phenotype compared to the other breast cancer subtypes but was not statistically significant. Notably, our results are similar to data in a recent study showing that positive PD-L1 immunostaining in locally advanced TNBC that received NAC was found to be 50% based on TCs and 46% based on ICs.⁶⁸

This variability in PD-L1 expression profiles can be attributed to several reasons. First, the use of different PD-L1-antibody clones can result in different staining patterns. Several PD-L1 antibody clones are available including E1L3N, E1J2J, 9A11, SP142, 28-8, 22C3, and SP263. In our work, we analyzed the immunohistochemical expression of PD-L1 using the 22C3 antibody clone, which was cleared by the FDA for the evaluation of PD-L1 immunorepression in TNBC patients receiving the PD-1 checkpoint inhibitor pembrolizumab.⁶⁹ Of note, a recent study which utilized three different antibody clones of PD-L1 in IDC, including the IHC 22C3 clone, has showed similar staining patterns between the three primary antibodies, suggesting that the expression level is not necessarily affected by the antibody clone used.⁷⁰ Second, the variation can be attributed to different cut-off values for evaluating PD-L1 positivity. Kim et al. used a cut-off of $\geq 5\%$ and identified a high positivity rate of PD-L1 expression of 64.7%,⁷¹ while Asano et al. used a cut-off of $\geq 10\%$ and identified a lower positivity rate of 23.7%.⁶¹ In our analysis, the cut-off for positivity was $\geq 1\%$ which was based on the antibody manufacturer's instructions and in consistence with the vast majority of previous studies.^{55,58,72,73} Third, a previous study suggested that prolonged storage (≥ 3 years) of FFPE tumor specimens

can affect PD-L1 immunorepression,⁷⁴ suggesting that differences in expression might be due the possibility of false-negative staining in older samples. However, another report confirmed that duration of storage of FFPE tumor specimen have minimal or no effect on PD-L1 immunostaining.⁷⁵ Moreover, we observed no difference in the intensity of PD-L1 staining between the positive control tissue samples (human tonsil and placenta tissue) which were stored for a prolonged period (≥ 10 years) and recently collected breast tumor samples. Lastly, and most importantly, our work was performed under strict inclusion/exclusion criteria evaluating PD-L1 expression level in IDC samples in patients with poor pathological response to NAC (mainly TAC regimen) without hormonal or radiation therapy prior to surgery, providing a more focused PD-L1 expression analysis in this subpopulation of patients. On that note, previous evidence suggested that breast cancer patients who had PD-L1 tumor cell overexpression prior to exposure to NAC exhibit a higher chance of achieving a complete pathological response (pCR).^{58,71,76} Moreover, the utilization of PD-1/PD-L1 checkpoint inhibitors in combination with chemotherapy was shown to provide better treatment outcomes in early TNBC including an increase of pCR rates compared to chemotherapy alone,⁷⁷ indicating that PD-L1 expression on neoplastic cells is of major therapeutic relevance.⁷⁸ Furthermore, a recent study demonstrated that the enhanced PD-L1 expression on immune cells following atezolizumab in combination with carboplatin plus nab-paclitaxel in TNBC patients, increased the chance to achieve a pCR.⁷⁹ Accordingly, it is possible that the relatively lower positive expression rates of PD-L1 post-NAC in our analysis are attributed to the fact that all studied samples were derived from patients with incomplete or partial PR to NAC. Lastly, in addition to evaluating PD-L1 expression level in TCs, we also measured its expression in ICs. According to previous studies, PD-L1 expression rate ranged between 16.2%–93% in the tumor microenvironment in breast cancer patients who did not receive NAC before surgery.^{63,76,80–82} Indeed, the increased immunorepression of PD-L1 in infiltrating ICs is of relevance as it was linked to higher potential of developing pCR in breast cancer compared to PD-L1 negative.^{58,83} In our analysis, PD-L1 expression rate was 31.7% (19/60) in non-neoplastic cells in the breast tumor microenvironment following NAC administration which is consistent with the previous study by Pelekanou et al.²² where PD-L1 expression rate in infiltrating ICs following NAC was 33% (14/43) in residual invasive breast cancer. Its noteworthy that our work has some limitations including a relatively small sample size, a small number of matching core-needle biopsies, and the use of only one antibody clone against PD-L1.

Over the past decades, the role of TILs in breast cancer has been studied in detail. Previous studies demonstrated

that high levels of TILs in pre-NAC biopsy samples are an established predictor of NAC response in breast cancer.^{84–86} However, Ochi et al. evaluated TILs levels pre and post-NAC in the 55 of TNBC and 75 HER-2 positive breast cancer,⁸⁷ revealing no significant difference in the TILs levels pre and post-NAC. On the contrary, a previous study demonstrated a significant decrease of TILs in the residual breast cancer following NAC.⁸⁸ In fact, our analysis in matched pre-NAC and post-NAC specimens showed lower TILs levels were found in the post-NAC resection specimens (median 1%) compared to the pre-NAC biopsy specimens (median 5%) despite not being statistically significant. Although a number of previous studies have shown an association between PD-L1 and TILs in breast cancer,^{27–29} we did not find a significant association between PD-L1 immune expression based on the three scoring approaches and the status of TILs.

Lastly, we found that the PD-L1 concordance rate among the three scoring approaches was 75%. Interestingly, Guo et al. showed that the PD-L1 concordance rate using TPS, ICS, and CPS scoring approaches reached 76% in triple-negative breast cancer patients.⁴⁴ Consistent with the other published studies,^{89,90} we found that PD-L1 expression in TCs, ICs, and CPS does not correlate with overall survival in breast cancer. Although it did not reach statistical significance, patients with PD-L1 negative tumors according to TPS score tended to demonstrate improved overall survival ($p = 0.051$).

Conclusions

Collectively, our work shows a significant decrease in PD-L1 immunoeexpression in ICs based on ICS in post-NAC specimens (McNemar's, $p = 0.020$), while no significant differences based on TPS and CPS in paired pre- and- post NAC samples ($p = 1.000$, $p = 0.617$; respectively). In post-NAC resection specimens, PD-L1 levels were more frequently expressed in ICs than in TCs or CPS (31.7% vs. 18.3% or 25; respectively). However, there was a low PD-L1 expression level in tumor samples following NAC exposure, suggesting that low PD-L1 expression might be a feature of breast cancer responding poorly to NAC. Our data suggests that further investigation of PD-L1 expression is required based on the PR to chemotherapy prior to the initiation of immunotherapy.

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Declaration of conflicting interests

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Ethics approval

Ethical approval for this study was obtained from *the Internal Review Board committees at both JRMS (ID No. 9/2019) and PHH (ID No. 32/2778).

Informed consent

Written informed consent was obtained from all subjects retrospectively.

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