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PD-L1 expression correlates with tumor-infiltrating lymphocytes and response to neoadjuvant chemotherapy in breast cancer

Hallie Wimberly[1], **Jason R Brown**[1], **Kurt Schalper**[1], **Herbert Haack**[2], **Matthew R. Silver**[2], **Christian Nixon**[1], **Veerle Bossuyt**[1], **Lajos Pusztai**[3], **Donald R Lannin**[4], and **David L Rimm**[1]

[1]Department of Pathology; Yale University School of Medicine, New Haven, CT

[2]Cell Signaling Technology, Inc.

[3]Department of Medical Oncology; Yale University School of Medicine, New Haven, CT

[4]Department of Surgery; Yale University School of Medicine, New Haven, CT

Abstract

Programmed death 1 ligand 1 (PD-L1) is an immune regulatory molecule that limits antitumor immune activity. Targeting of PD-L1 and other immune checkpoint proteins has shown therapeutic activity in various tumor types. The expression of PD-L1 and its correlation with response to neoadjuvant chemotherapy in breast cancer has not been studied extensively. Our goal was to assess PD-L1 expression in a cohort of breast cancer patients treated with neoadjuvant chemotherapy. Pre-treatment biopsies from 105 breast cancer patients from Yale New Haven Hospital that subsequently received neoadjuvant chemotherapy were assessed for PD-L1 protein expression by automated quantitative analysis (AQUA) with a rabbit monoclonal antibody (E1L3N) to the cytoplasmic domain of PD-L1. Additionally, tumor-infiltrating lymphocytes (TIL) were assessed on H&E slides.PD-L1 expression was observed in 30% of patients and it was positively associated with hormone-receptor negative and triple-negative status and high levels of TILs. Both TILs and PD-L1 measured in the epithelium or stroma predicted pathologic complete response (pCR) to neoadjuvant chemotherapy in univariate and multivariate analysis. However, since they are strongly associated, TILs and PD-L1 cannot both be included in a significant multivariate model.PD-L1 expression is prevalent in breast cancer, particularly hormone-receptor negative and triple-negative patients, indicating a subset of patients that may benefit from immune therapy. Furthermore, PD-L1 and TILs correlate with pCR and high PD-L1 predicts pCR in multivariate analysis.

Keywords

PD-L1; neoadjuvant chemotherapy; breast cancer; predictive biomarkers; tumor-infiltrating lymphocytes

Corresponding Author: David L Rimm, MD, PhD, Department of Pathology, Yale University School of Medicine, BML116, 310 Cedar Street, PO Box 208023, New Haven, CT 06520-8023, USA; david.rimm@yale.edu.

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INTRODUCTION

Neoadjuvant chemotherapy is increasingly used in the management of stage II-III breast cancer and pathologic complete response (pCR) is observed in 5-15% of estrogen-receptor (ER) positive and 30-50% of triple-negative (TNBC) and HER2 positive patients with third generation combination chemotherapy regimens (1, 2). High-grade and high Ki67 expression also correlates with pCR particularly among ER positive cancers (3). Recent reports have found that the presence of tumor-infiltrating lymphocytes (TIL) predicts response to neoadjuvant chemotherapy (4-13). The presence of TILs may indicate immune-mediated host defense against the tumor, and TILs may contribute to and augment chemotherapy-induced cell death. The recent positive results with immune checkpoint inhibitors in melanoma and lung cancer have stimulated new interest in TILs and their relationship to tumor immunity and chemotherapy response (14, 15).

One key immune modulatory pathway is mediated by the PD-1/PD-L1 axis (Programmed cell Death 1 and its ligand). PD-L1 is a transmembrane protein of the B7 family of immune molecules that plays an integral role in limiting the cytotoxic immune response via interaction with programmed death-1 (PD-1) receptor (16). PD-L1 expression has been noted in a variety of cancers and reported in a variety of solid tumor types, including lung, melanoma, ovarian, colon and breast.(16-19) Its expression in tumor cells or presence in the tumor microenvironment has been correlated to the presence of TILs. Results from various preclinical studies using cell line and mouse models support the idea that inhibition of the interaction between PD-L1 and PD-1 in the tumor microenvironment may enhance antitumor immunity and promote tumor regression (16, 20-22). Various agents targeting PD-1 or PD-L1 are currently in clinical trials for a variety of solid tumor types and have demonstrated robust response rates, notably in metastatic melanoma, renal cell carcinoma and non-small cell lung cancer.(15, 23-28)

Our goal was to investigate the correlation of PD-L1, also known as B7-H1 and CD274, with TILs and pCR following neoadjuvant chemotherapy in breast cancer. We assess PD-L1 expression objectively by quantitative immunofluorescence on samples from a cohort of breast cancer patients that received neoadjuvant chemotherapy. We describe localization and distribution of PD-L1 expression and relate this to TILs, clinical characteristics of the cancer and response to neoadjuvant chemotherapy.

MATERIALS AND METHODS

Patient cohort

The cohort used in this study consists of 94 pre-surgical biopsies from patients diagnosed with breast cancer between 2002 and 2010, who subsequently received neoadjuvant chemotherapy. Specimens were collected from the archives of the Department of Pathology at Yale University. A majority of patients (76.6%) received adriamycin-based neoadjuvant chemotherapy. A more detailed characterization of the Yale Neoadjuvant Cohort has been published previously and is shown in Table 1 (3). pCR was defined as the absence of invasive carcinoma in the breast and sampled lymph nodes ypT0 ypN0.

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Evaluation of TILs

Histopathologic analysis of TILs was performed on hematoxylin and eosin (H&E)–stained sections from the core biopsies of 94 patients from the cohort described above. Analysis was conducted by two pathologists (V.B. and C.N.), who were blinded to the clinical parameters and response. TILs were quantified as a percentage estimate of the stromal area adjacent to the tumor that contained lymphocytic infiltrate, as described in the literature (6). Percentages were reported in discrete increments of 10 percent, with 0 percent indicating a minimal infiltrate and 100 percent indicating the stroma almost exclusively consisted of TILs. Sections with 50 percent or greater TIL infiltrate were denoted as lymphocyte predominant breast cancer (LPBC) as discussed in the literature (4).

Quantitative Immunofluorescence

Whole-tissue sections were baked overnight at 60°C then soaked in xylene twice for 20 min each. Slides were rehydrated in two- 1 minute washes in 100% ethanol followed by 1 wash in 70% ethanol and finally rinsed in streaming tap water for 5 minutes. Antigen retrieval was performed in sodium citrate buffer, pH6 in the PT module from LabVision. Endogenous peroxidases were blocked by 30 minute incubation in 2.5% hydrogen peroxide in methanol. Subsequent steps were carried out on the LabVision 720 Autostainer (Thermo-Scientific). Non-specific antigens were blocked by a 30 minute incubation in 0.3% BSA in TBST. Primary PD-L1 (E1L3N) rabbit monoclonal antibody (Cell Signaling Technology, clone E1L3N; see Supplemental Fig 2 for antibody validation) was prepared to a working concentration of 3.5%g/ml combined with 1:100 pan-cytokeratin antibody (Dako, Cat#Z062201-2) in 0.3% BSA in TBST and transferred to 4°C overnight. Primary antibodies were followed by incubation with Alexa 546-conjugated goat anti-rabbit secondary antibody (Life Technologies, Cat#A-11010) diluted 1:100 in mouse EnVision reagent (Dako, Cat#K400111-2) for 1 hour. Signal was amplified with Cy5-Tyramide (Perkin Elmer, Cat#SAT705A001EA) for 10 minutes and then slides were mounted with ProlongGold + DAPI (Life Technologies, Cat#P36931).

Immunofluorescence was quantified using automated quantitative analysis (AQUA) on all regions of tissue on each slide. Briefly, fluorescent images of DAPI, Cy3 (Alexa 546-cytokeratin) and Cy5 (PD-L1) for each field of view were collected. The number of fields of view assessed per case ranged from 5 to 93. The average number of fields of view was 32. Image analysis was carried out using the AQUAnalysis software (Genoptix), which is generated for each compartment by dividing the sum of target pixel intensities by the area of the compartment in which the target is measured (29, 30). The stromal compartment was created by subtracting the epithelial tumor mask from a DAPI mask.

Statistical Analysis

T-tests were used to determine the correlations between continuous quantitative scores of PD-L1 expression and clinicopathologic factors as well as pCR. Chi-square tests were used to determine correlation of binary PD-L1 expression with pCR. Logistic regression was used for univariate and multivariate analyses. All statistical tests mentioned above were carried out using StatView (SAS Institute Inc.). Joinpoint software was used to dichotomize continuous PD-L1 AQUA scores. Briefly, average quantitative scores and the standard

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deviation for each patient with greater than four fields of view of tissue were imported into Joinpoint software, which identifies trends in the population distribution, enabling an objective method of splitting a population in two (31).

RESULTS

Of the 94 cases collected for the neoadjuvant cohort, 14.9% (14/94) and 19.1% (18/94) were eliminated from consideration for PD-L1 staining in the epithelium and stroma, respectively, due to insufficient measureable tissue. Our criteria for the minimum amount of tissue for evaluation are 4 fields of view per tissue section with a minimum of 3% area within the field of view of the epithelial or stromal compartment. The number of fields of view analyzed per biopsy ranged from 5-93 with an average of 34. We could assess epithelial PD-L1 expression in 80 cases and stromal PD-L1 expression in 76 cases. Examples of epithelial and stromal PD-L1 expression can be seen in Supplementary Fig 1C-F. Heat maps of AQUA scores generated on one whole tissue section in the epithelial and stromal compartments are shown in Supplementary Fig 1A,B, demonstrating a higher level of PD-L1 expression in the epithelium for the example given. The AQUA scores were reflective in each case of the predominant localization of PD-L1 expression and Fig 1A shows an example of the distribution of AQUA scores within a tissue section of epithelial-predominant expression while the distribution of a case with predominantly stromal PD-L1 expression is illustrated in Fig 1B. The distribution of PD-L1 expression in the epithelial and stromal compartments are similar (see Fig 1C,D), although epithelial expression showed higher signal, reflected by higher AQUA scores, than stromal expression (Fig 1C,D). While the distributions in Fig 1 C,D represent averages of scores from a number of fields of view from each patient, we note the heterogeneity of PD-L1 expression within each biopsy. Fig 1A,B illustrate that the level of PD-L1 expression can vary up to 4X in different areas of the same biopsy. When measured as a continuous quantitative score, high PD-L1 expression in epithelial cells or stroma is significantly associated with hormone-receptor negative and triple-negative breast cancers. Using a threshold adopted from the literature as described in the methods, 8.5% (8/94) of cases were LPBC. We also find a significant positive association of PD-L1 with LPBC (Table 2).

PD-L1 in the epithelium and stroma correlates with pCR when measured as a continuous quantitative score (Fig 1E,F; epithelial P-value=0.0189; stromal P-value=0.0050). Analysis of PD-L1 expression as a continuous quantitative score in subsets of patients that were LPBC-positive, hormone receptor-positive, HER2-amplified and triple-negative revealed that PD-L1 in the epithelium and stroma correlates with pCR only in hormone receptor-positive and HER2-amplified breast cancers, though analyses in LPBC and triple-negative subsets may be underpowered (Supplemental Fig 3).

In order to determine a statistically rigorous cut point of PD-L1 using the continuous quantitative data, Joinpoint software was used (31). As shown in Supplemental Fig 4, Joinpoint identified three differential points in the distribution of both epithelial (Supplemental sFig 4A) and stromal (Supplemental Fig 4B) continuous PD-L1 scores in the cohort, one of which located to the approximate visual threshold of PD-L1 positivity. We dichotomized PD-L1 at Joinpoint#2 for both epithelial and stromal scores, making

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approximately 30% of the cohort positive for PD-L1 expression. When dichotomized by Joinpoint software in this way, PD-L1 correlates with pCR (Supplemental Fig 5; epithelial Chi Square P-value=0.0595; stromal Chi Square P-value=0.0499).

Examples of H&E images from cases scored as non-LPBC, with TIL infiltrate less than 50 percent, and LPBC, with TIL infiltrate ≥ 50 percent, are shown in Fig 2A,B. Fig 2A represents a case scored as non-LPBC, with the two pathologists scoring <5 and 20% TIL component, respectively. Fig 2C is an image of PD-L1 staining, showing little to no reactivity in the same TIL low case. Fig 2B represents a case scored as LPBC, with the two pathologists scoring 70 and 80% TIL component. PD-L1 staining in the same case shows robust expression in the stroma (Fig 2D). Further, PD-L1 as measured on the entire cohort as a continuous quantitative score in the epithelium and stroma positively correlates with high TIL component (Fig 3A,B; epithelial P-value<0.0001; stromal P-value=0.0001).

Univariate analyses using logistic regression identified node status and LPBC as predictors of pCR to neoadjuvant chemotherapy (Table 3). Multivariate analyses including age, nodal status, tumor size, molecular classification (hormone therapy sensitive, HER-2 positive, or triple-negative), nuclear grade, Ki-67 AQUA score, TILs, and PD-L1 expression revealed that both epithelial and stromal PD-L1 expressions are nearing significance in predicting pCR in this multivariate analysis (Table 4). The p value becomes significant for both if Ki67 is excluded from the analysis (Supplemental Table 1).

DISCUSSION

PD-L1 expression in the epithelium or stroma as a continuous quantitative score or dichotomized into negative and positive predicts pCR. Dichotomized scores of epithelial or stromal expression are not independent of one another, though individually both are predictive of pCR in a multivariate model including age, nodal status, tumor size, hormonal receptor status, HER2 and triple-negative status. PD-L1 expression in the epithelium and stroma is associated with ER- and PR-negative, triple-negative, and LPBC breast cancers.

The evaluation of PD-L1 expression is challenging due to heterogeneity in expression and non-reproducibility of antibody reagents (32). Only a few studies have described PD-L1 protein expression in breast cancer patients, finding an association of PD-L1 with proliferative markers and FoxP3 T-regulatory cells (18, 33). We found that roughly 30% of breast cancers express PD-L1 in the epithelium and/or stroma. The heterogeneous nature of PD-L1 expression in breast cancer, being both epithelial and/or stromal as well as present in only select fields of view, supports previous studies that suggest its expression is limited to specific regions of the tumor such as the invasive front (17). Previous studies have also described localization in both epithelial cells and on specific cells in the stroma. Our work confirms expression in both compartments, but we found no differences in epithelial or stromal expression with respect to prediction of pCR or association with various clinical features.

In addition to predicting response to neoadjuvant chemotherapy, we investigated whether PD-L1 expression may also be a biomarker for predicting response to immune therapies

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targeting the PD-1/PD-L1 pathway. Indeed, a landmark Phase I trial of an anti-PD-1 antibody on various solid tumor types showed that the only patients with objective response to anti-PD-1 therapy were those whose tumors expressed PD-L1 (15). A limitation thus far in the assessment of PD-L1 protein expression has been a lack of specific and reproducible antibodies for use on formalin-fixed paraffin-embedded tissue. Of the eight commercially available antibodies, only 3, including the E1L3N clone from Cell Signaling Technology used in this study passed our quality control (data not shown). In previous studies we have examined PD-L1 expression using clone 5H1 through a collaboration with Lieping Chen (Dept of Immunobiology, Yale University) (32). The antibody used in this study to detect PD-L1 shows similar, but not identical staining compared to clone 5H1 (see Supplemental Fig 2C-E). This clone is commercially available and uses a more standardized staining protocol and yields reproducible results.

While we believe this study illustrates the value of PD-L1 as a potential prognostic marker, there are a number of limitations. This is a retrospective study comprising a modest sample size of patients from a single institution. While the majority of patients (76.6%) received anthracycline-based neoadjuvant therapy, the remainder received variations of taxanes and/or carboplatin, limiting any interpretation of treatment-specific results. Another limitation is that only a single monoclonal antibody was used to assess PD-L1 expression. While this is considered acceptable in a publication, efforts are underway to evaluate other validated antibodies. We have measured PD-L1 mRNA expression on tissue microarrays containing a large number of breast cancer samples, although not yet on this neoadjuvant cohort (34). Furthermore, the potential information contained within the dynamic nature of PD-L1 expression (heterogeneity of localization as well as intensity of expression) may be over-simplified by our methods of analysis. Finally, this cohort is too recent to provide a meaningful prognostic evaluation of PD-L1 expression. While TILs have been reported to be prognostic and PD-L1 expression correlates with the presence of TILs, the prognostic value of PD-L1 will be assessed in future work.

The close correlation of PD-L1 with TILs and the ease with which PD-L1 can be induced by expression of inflammatory cytokine interferon-γ suggests that PD-L1 may act as a surrogate marker for an antitumor immune response, albeit one that is being down-regulated. The presence of both TILs and PD-L1 in the tumor microenvironment could indicate an adaptive immune resistance to endogenous antitumor activity, suggesting that patients with both of these components would benefit from immunotherapy (17).

In summary, we demonstrate a reproducible assay for evaluating PD-L1 protein expression on formalin-fixed, paraffin-embedded tissue sections that predicts response to neoadjuvant chemotherapy, independent of localization and treatment. PD-L1 expression also correlates with the presence of TILs. However, the value of this marker will be its use in patients treated with PD-L1 axis-directed therapies. In the future, we look forward to using these same reagents and methods to evaluate treated patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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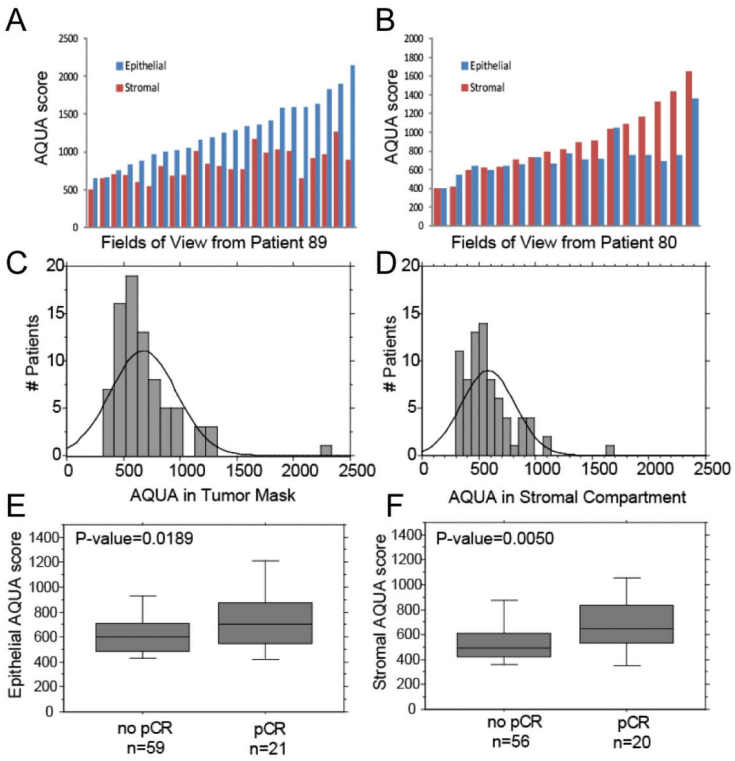

Fig 1.

Epithelial and stromal PD-L1 expression correlates with pathologic complete response (pCR). (A,B) Distributions of epithelial and stromal PD-L1 expression with quantitative scores for each field of view in two representative cases. A frequency distribution of the epithelial (C) and stromal (D) PD-L1 quantitative scores. Box plots with continuous PD-L1 scores in epithelium (E) and stroma (F) on the Y-axis and pCR or no pCR on the X-axis.

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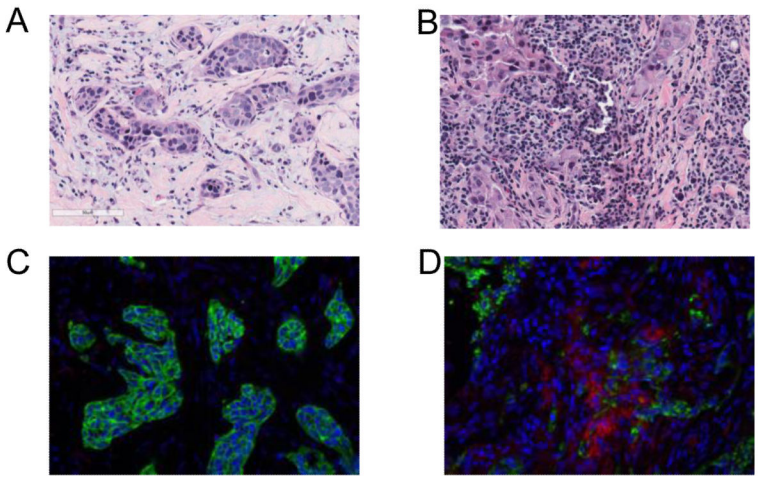

Fig 2.

Examples of PD-L1 expression in breast cancers with low and high levels of TILs. Representative H&E and PD-L1 in cases scored non- lymphocyte predominant breast cancer (LPBC) (A,C) and LPBC (B,D). Immunofluorescence images blue=DAPI, green=pan-cytokeratin, red=PD-L1.

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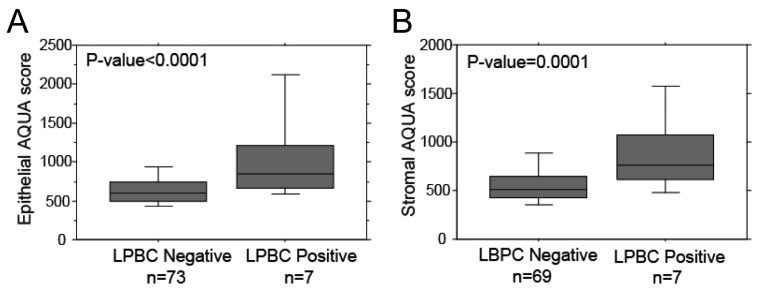

Fig 3.

Quantitative assessment of epithelial and stromal PD-L1 expression correlates with TILs. Box plots with continuous PD-L1 scores in epithelium (A) and stroma (B) on the Y-axis and lymphocyte predominant breast cancer (LPBC) or non-LPBC on the X-axis.

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Table 1

Yale Neoadjuvant Cohort Characteristics

Characteristic	N	%
Age (y)		
<50	55	58.5
≥50	39	41.5
Unknown	0	0
Nodal Status		
Positive	48	51.1
Negative	32	34.0
Unknown	14	14.9
Tumor Size (cm)		
<2	10	10.6
>2	83	88.3
Unknown	1	1.1
Nuclear Grade		
1-2	48	51.1
3	43	45.7
Unknown	3	3.2
ER		
Negative	35	37.2
Positive	57	60.7
Unknown	2	2.1
PR		
Negative	42	44.7
Positive	50	53.2
Unknown	2	2.1
HER2		
Negative	68	72.3
Positive	25	26.6
Unknown	1	1.1
Triple Negative		
Yes	23	24.4
No	70	74.5
Unknown	1	1.1
LPBC		
Yes	8	8.5
No	84	89.4
Unknown	2	2.1
Treatment		
Adriamycin-based	72	76.6
Carboplatin+Taxane+Herceptin	10	10.6

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Characteristic	N	%
Carboplatin+Abraxane+Avastin	6	6.4
Carboplatin+Abraxane+Herceptin	3	3.2
Carboplatin+Taxol+Etoposide	1	1.1
Cytoxan+Taxotere	2	2.1

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Table 2

Epithelial and stromal PD-L1 expression association with Yale Neoadjuvant Cohort characteristics

Characteristic	P-Value	
	Tumor Mask	Stroma
Age (y)		
<50	0.3036	0.4228
≥50		
Nodal Status		
Positive	0.3062	0.3811
Negative		
Tumor Size (cm)		
<2	0.4708	0.8819
>2		
ER		
Negative	0.0378*	0.0219*
Positive		
PR		
Negative	0.0099*	0.0009*
Positive		
HER2		
Negative	0.7381	0.7826
Positive		
Triple Negative		
Yes	0.0026**	0.0021**
No		
LPBC		
Yes	<0.0001**	<0.0001**
No		

* negative association with PD-L1

** positive association with PD-L1

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Table 3

Univariate analysis of likelihood of pCR on Yale Neoadjuvant Cohort

	Univariate		
Variable	**Odds Ratio***	**95% CI**	**P-value**
Age (y)			
<50	1		
≥50	0.923	0.377-2.259	0.8609
Nodal Status			
Positive	0.126	0.042-0.379	**0.0002**
Negative	1		
Tumor Size (cm)			
<2	1		
>2	0.383	0.101-1.449	0.1575
Nuclear Grade			
1-2	1		
3	2.485	0.983-6.278	0.0543
ER			
Negative	1		
Positive	0.488	0.197-1.209	0.1211
PR			
Negative	1		
Positive	0.415	0.167-1.030	0.0579
HER2			
Negative	1		
Positive	2.357	0.901-6.167	0.0806
Triple Negative			
No	1		
Yes	2.222	0.831-5.941	0.1115
LPBC			
No	1		
Yes	4.697	1.036-21.302	**0.0449**
Epithelial PD-L1			
Low	1		
High	2.667	0.945-7.528	0.0639
Stromal PD-L1			
Low	1		
High	2.804	0.982-8.006	0.0541

* Odds Ratio indicates likelihood of pCR

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Table 4

Multivariate analysis of likelihood of pCR on Yale Neoadjuvant Cohort

	Multivariate (TIL)			Multivariate (Epithelial PD-L1)			Multivariate (Stromal PD-L1)		
Variable	**Odds Ratio***	**95% CI**	**P-value**	**Odds Ratio***	**95% CI**	**P-value**	**Odds Ratio***	**95% CI**	**P-value**
Age (y)									
<50	1			1			1		
≥50	0.482	0.086-2.711	0.4077	0.164	0.011-2.329	0.1815	0.147	0.009-2.290	0.1713
Nodal Status									
Negative	1			1			1		
Positive	0.026	0.003-0.219	**0.0008**	0.009	0.0003-0.254	**0.0057**	0.009	0.0003-0.284	**0.0072**
Tumor Size (cm)									
<2	1			1			1		
>2	0.110	0.014-0.841	**0.0334**	0.054	0.004-0.676	**0.0236**	0.072	0.006-0.915	**0.0425**
Nuclear Grade									
1-2	1			1			1		
3	4.780	0.766-29.822	0.0939	2.920	0.225-37.903	0.4126	3.105	0.238-40.520	0.3872
Molecular Category									
HR+/HER2 −	1			1			1		
HER2+	2.908	0.390-21.704	0.2979	3.624	0.172-76.513	0.4079	4.156	0.206-83.696	0.3524
Triple Negative	0.614	0.061-6.167	0.6783	0.705	0.046-10.724	0.8013	0.864	0.057-13.138	0.9160
Ki-67									
Low	1			1			1		
High	6.186	0.838-45.651	0.0739	7.945	0.530-119.073	0.1335	8.550	0.570-128.29	0.1204
LPBC									
No	1								
Yes	6.019	0.035-1029.2	0.4938						
Epithelial PD-L1									
Low				1					
High				11.120	0.870-142.159	0.0639			
Stromal PD-L1									
Low							1		
High							11.267	0.779-162.95	0.0756

* Odds Ratio indicates likelihood of pCR