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Original Article

Combining Analysis of Tumor-infiltrating Lymphocytes (TIL) and PD-L1 Refined the Prognostication of Breast Cancer Subtypes

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Abstract

Background: PD-L1 has been used as a biomarker to select patients for treatment of PD-1/PD-L1 inhibitors.

Materials and Methods: In this study, we assessed the clinicopathological features of breast cancers that are associated with PD-L1 expression, as well as its relationship with other immune components and its prognostic significance.

Results: Totally 1752 cases were included in this cohort. PD-L1 expression in tumor-infiltrating immune cells (PD-L1-IC) expression and in tumor cells (PD-L1-TC) expression were identified in 34.2% and 10.1% of cases, respectively, and they showed a positive correlation with higher tumor grade, morphological apocrine features, presence of necrosis, and higher stromal tumor-infiltrating lymphocytes (sTIL). PD-L1-IC and PD-L1-TC expression correlated positively with each other, and both of them were negatively associated with estrogen receptor and progesterone receptor and positively associated with Ki67, HER2, EGFR, p63, and *p*-cadherin. In survival analysis, PD-L1-IC expression was associated with better disease-free survival (DFS) and breast cancer-specific survival (BCSS) in HER2-overexpressed (HER2-OE) cancers and high–grade luminal B cancers. In triple–negative breast cancers (TNBC) and HER2–OE cancers, compared with sTIL low PD-L1-IC negative cases, sTIL high cases showed significantly better DFS independent of PD-L1-IC status. sTIL low PD-L1-IC positive cases also demonstrated a better DFS in HER2–OE cancers. In high–grade luminal B cancers, sTIL high PD-L1-IC positive cases showed the best BCSS.

Conclusion: The data suggested that the combining analysis of sTIL and PD-L1-IC expression refined the prognostication of breast cancer subtypes. Cases with high TIL and PD-LI-IC expression appear to be more immune active.

Key words: breast cancer; tumor microenvironment; PD-L1; stromal tumor-infiltrating lymphocytes.

Implications for Practice

PD-L1-IC expression was associated with better disease-free survival (DFS) and breast cancer-specific survival (BCSS) in HER2–overexpressed (HER2-OE) cancers and high–grade luminal B cancers. In triple–negative breast cancers (TNBC) and HER2–OE cancers, compared with sTIL low PD-L1-IC negative cases, sTIL high cases regardless of PD-L1-IC status showed significantly better DFS. sTIL low PD-L1-IC positive cases also demonstrated a better DFS in HER2–OE cancers. In high–grade luminal B cases, sTIL high PD-L1-IC positive cases showed the best BCSS. The data suggested that the combining analysis of sTIL and PD-L1-IC expression refined the prognostication of breast cancer subtypes.

Introduction

Following surgical resection, the mainstay treatment for breast cancer includes a combination of radiotherapy, chemotherapy, and hormonal therapy. Additional anti–HER2 target therapy is given to those with HER2–positive cancers. With these treatment regimens, majority of patients can achieve long–term survival. Unfortunately, 5-11% of patients eventually present with metastatic disease, and in a significant fraction of patients, the tumors are resistant to systemic treatment and these patients will eventually develop distant relapses and ultimate mortality.[1-3]

It is now well recognized that tumor microenvironment plays a crucial role in the development of cancer. Cancer immune condition has been revealed as a major hallmark of cancer. Recent advancement in cancer immunology has revolutionized cancer treatment and prognostication. Immunotherapy harnessing the immune system's natural ability to fight against cancer cells has received increasing attention. The most promising immunotherapeutic approach developed is the immune checkpoint blockade (ICB). The immune checkpoints are upregulated upon continued immunologic stimulus, acting

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as a negative regulator to dampen the immune response. Cancers can hijack this network to circumvent the anti-cancer immunity. Currently approved checkpoint inhibitors target the molecules CTLA4, PD-1, and PD-L1. Improved outcome of ICB has been observed in various malignancies, including melanoma, urothelial cell carcinoma, renal cell carcinoma, and non–small-cell lung cancer.[3-6]

Although breast cancer has been considered as an "immune-cold" tumor, a proportion of them could be "inflamed," in particular the more aggressive subtypes, showing a high level of tumor-infiltrating lymphocytes and tumor mutational burden.[7] There is a great interest in exploring the potential role of immunotherapy in breast cancer. The results from the Phase III Impassion 130 trial marked a new milestone in breast cancer treatment.[8] The addition of atezolizumab, a PD-L1 inhibitor, to nab-paclitaxel in the first-line treatment of incurable, locally advanced or metastatic triple-negative breast cancer (TNBC) prolonged progression-free survival. Furthermore, in the PD-L1-positive subgroup, which was detected by the Ventana PD-L1 SP142 immunohistochemistry (IHC) assay, overall survival was improved in the atezolizumab treatment arm. Atezolizumab, thus, has been approved by FDA in 2019 for the metastatic TNBC tumors with PD-L1 positivity and SP142 assay as the companion diagnostic test. However, primary results from the clinical trial Impassion131 showed that combining atezolizumab with paclitaxel did not improve progression-free survival or overall survival versus paclitaxel along (https://doi.org/10.1016/j.annonc.2021.05.801), resulting in the recent voluntary withdrawal of breast cancer indication from atezolizumab in the US.

This discrepancy indicates stratification based on PD-L1 expression may not be sufficient.

Data from melanoma and lung cancers showed that the patients with "PD-L1–positive" tumors had an overall response rate of 48% to ICB, whereas 15% of patients responded despite PD-L1 negativity.[9] Beyond PD-L1 expression, recent attention has been shifted to the tumor genome and neoantigen, phenotype of tumor immune status, and other host–related features for treatment prediction. Biomarkers, such as the density of tumor-infiltrating T cells, immune cell profiles, MHC class I expression and the tumor mutational burden are under consideration. Their expression alone or together with PD-L1 has been examined for their association with treatment response.[10] Regarding breast cancer, little has been reported on PD-L1 expression by SP142 assay and its relationship with other immune or host factors. In addition, its clinicopathological analysis was focused on TNBC cancers,[11-15] and relatively little was known for the other breast cancers. With the success of ICB in metastatic TNBC, trials have been conducted with ICB also in other subtypes.[16] The purpose of this retrospective study is to assess the clinical and pathological features of breast cancers that are associated with PD-L1 (SP142) expression in the tumor cells and stromal tumor-infiltrating immune cells in a large breast cancer cohort with different breast cancer subtypes, as well as the relationship between PD-L1 expression and other immune components.

Materials and Methods

Patients Data

All consecutive cases diagnosed with breast cancer over a period of 4 (2002-2005), 7 (2003-2009), and 4 (2003-2006) years in 3 of the involved institutions were included. The cases with neoadjuvant therapy were excluded. Patients' demographic data (age), histopathologic parameters (tumor size, lymph node involvement, pN stage, and pT stage) and outcome data were retrieved from the medical records. Disease-free survival (DFS) time was calculated from the date of the surgery to the date of the first relapse or death. Breast cancer-specific survival (BCSS) time was calculated from the date of the surgery to the date of dying from breast cancer. All the specimens were fixed in 10% buffered formalin and embedded in paraffin. Archival H&E stained slides for each case were reviewed to confirm the diagnosis (WHO criteria) and grade (Bloom and Richardson grading). Stromal tumor-infiltrating lymphocytes (sTIL) were evaluated based on the percentage of tumor-stromal area occupied by TIL (International Immuno-Oncology Biomarker Working Group on Breast Cancer) on whole sections. TIL level >20% was considered as sTIL high, and TIL level <=20% was considered as sTIL low.[17] Additional histologic features [including lymphovascular invasion (LVI), morphological apocrine feature, fibrotic change, necrosis, and extensive in situ components (EIC)] were assessed as previously reported.[18] The study was approved by the Joint Chinese University of Hong Kong—New Territories East Cluster clinical research ethics committee. Tissue from patients was acquired with informed consent in accordance with local institutional review and the Declaration of Helsinki.

Tissue Microarray Construction and Immunohistochemistry

Tissue microarray (TMA) was prepared as previously described.[18] Briefly, representative tumor areas of each case were selected and 0.6 mm core in duplicate was taken for TMA construction. The presence of tumor was confirmed on H&E stained TMA sections. Immunohistochemical (IHC) staining was carried out on TMA sections with the selected antibodies using Ultraview Universal DAB Detection Kit (Ventana, Arizona, USA) after deparaffinization, rehydration, and antigen retrieval of the slides. All slides were counterstained with hematoxylin. The IHC staining was evaluated based on staining intensity (graded from 0 to 3) and the percentage of positively stained cells in the corresponding cellular location according to different antibodies. The interpretation of IHC results was carried out blindly by 2 of the authors without any clinical information and the staining results of other markers. Any discrepancies were resolved by discussion to reach a consensus. PD-L1 expression in tumor cells (PD-L1-TC) and in tumor-infiltrating immune cells (PD-L1-IC) were assessed as previously reported.[19] Briefly, PD-L1-TC was assessed as the proportion of tumor cells showing membrane staining of any intensity: PD-L1-TC negative (<1%) and PD-L1-TC positive (≥1%); PD-L1-IC was assessed as the proportion of tumor area occupied by PD-L1-positive IC of any intensity: PD-L1-IC negative (<1%) or PD-L1-IC positive (≥1%). Tumor area was defined as the area containing viable TC, their associated intratumoral stroma and contiguous peritumoral stroma. Results for other biomarkers, including estrogen receptor (ER), progesterone receptor (PR), HER2, Ki67, EGFR, c-Kit, p63, CK5/6, vimentin, p-Cadherin, AR, HVEM, PD1 TIL, HLA-A, HLA-B, and HLA-C were retrieved from our database.[18,20,21] Details of staining and assessment of all markers involved in the study are shown in Supplementary Table S1.

The tumors were also classified into different molecular subtypes: luminal A (ER+, PR ≥ 20%, HER2–, Ki67 < 20%), luminal B (ER+, PR < 20% and/or HER2+ and/or Ki67 ≥ 20%), HER2-overexpressed (HER2-OE) (ER–, PR–, HER2+), and triple-negative breast cancers (TNBC) (ER–, PR–, HER2–) (including basal-like breast cancers (BLBC) (ER–, PR–, HER2–, CK5/6+, and/or EGFR+) and 5-marker negative panel (5NP) (ER–, PR–, HER2–, CK5/6–, EGFR–)) using IHC results as surrogates.

Statistical Analysis

SPSS for Windows (version 26.0; SPSS Inc., Chicago, IL) was used for all statistical analyses. Chi-square analysis or Fisher's exact test were used to test the association between categorical variables. Survival data were analyzed using the Kaplan-Meier method and group differences in survival time were investigated by a log-rank test. Multivariate Cox proportional hazards model with backward Wald model were used to identify variables that were independently associated with survival. All statistical tests were 2-sided, and *P*-value of <.05 was considered statistically significant.

Results

In total, 1752 primary breast cancers were included in this study. The mean patients' age at diagnosis was 54.1 ± 12.8 years (range 22-101 years) and the mean tumor size was 2.66 ± 1.47 cm (range 0.1-13.0 cm). There were 232 (13.2%), 727 (41.5%), and 793 (45.3%) of grades I, II, and III, respectively. ER, PR, and HER2 were positive in 69.9% (1225/1740), 67.4% (1171/1737), 18.9% (329/1742) of the cases, respectively. Based on IHC surrogates for molecular subtyping, there were 683 (39.4%), 628 (36.2%), 163 (9.4%), 259 (15.0%) cases of luminal A, luminal B, HER2-OE, and TNBC subtypes, respectively. In luminal B cases, 143 (22.7%) were HER2 positive and 485 (77.3%) were HER2 negative. Among those, 310 cases were high–grade luminal B (luminal grade 3), 88 of them (28.4%) are HER2 positive and 222 of them (71.6%) are HER2 negative.

PD-L1-IC expression (Fig. 1) was identified in 34.2% (600/1752) of cases, with the highest expression rate of 54.1% (140/259) in TNBC. PD-L1-TC expression (Fig. 2) was detected in 10.1% (173/1711) of cases, with the highest expression rate of 20.3% (33/162) in the HER2–OE subtype, followed by TNBC (11.6%; 29/252) (Tables 1 and 2).

Correlation with Clinico-pathological Features, Biomarkers, and Breast Cancer Molecular Subtypes

PD-L1-IC expression is more likely to be found in younger patients (P = .007). It showed a positive correlation with higher tumor grade (P < .001), morphological apocrine features (P = .005), presence of necrosis (P < .001), higher sTIL (P < .001), higher T stage (P < .044), and higher N stage (P = .019), and a negative correlation with fibrotic focus (P = .016) and extensive intraductal carcinoma (P = .001) (Table 1). Similarly, PD-L1-TC expression is associated positively with higher tumor grade (P = .003), morphological apocrine features (P < .001), presence of necrosis (P < .001), and higher sTIL (P < .001) (Table 2).

For biomarker expression, PD-L1-IC and PD-L1-TC expression correlated positively with each other (P = .011). Positive correlations of PD-L1-IC were found with ki67 expression, HER2, EGFR, C-KIT, p63, CK5/6, CK14, vimentin, and p-cadherin (P < .001 for all), but negatively with ER, PR, and AR (P < .001 for ER and PR, P = .034 for AR). Similarly, PD-L1-TC expression was also positively associated with Ki67, HER2, EGFR, p63, and p-cadherin (P ≤ .002) and negatively with ER and PR (P ≤ .026). Unlike PD-L1-IC, no significant correlation was found with c-kit, CK5/6, CK14, vimentin, and AR (Table 2).

For breast cancer molecular subtypes, both PD-L1-IC expression and PD-L1-TC expression showed a differential expression among different molecular subtypes (P < .001 for both), with higher levels in HER2-OE/TNBC and the least in luminal A cancers (Tables 1 and 2).

The clinicopathological characteristics of PD-L1-IC and PD-L1-TC were further explored in 3 aggressive breast cancer subtypes, namely high–grade luminal B (grade 3 luminal B), HER2-OE, and TNBCs. In high–grade luminal B, PD-L1-IC expression was associated positively with the presence of morphological apocrine features, high level of sTIL, the expression of HER2, CK5/6, CK14, HVEM, HLA-A, HLA-B, HLA-C, combined HLAs expression status, and PD1+TIL (P ≤ .036), while PD-L1-TC was only associated with the presence of apocrine phenotype (P = .011). For HER2-OE, PD-L1-IC expression was associated positively with high-level of sTIL, the expression of HLA-A, HLA-B, HLA-C, combined HLAs expression status and PD1+TIL (P ≤ .041), while PD-L1-TC was only associated with high ki67 (P = .021). Among the TNBCs, PD-L1-IC expression was associated positively with higher grade, high level of sTIL, the presence of necrosis, the

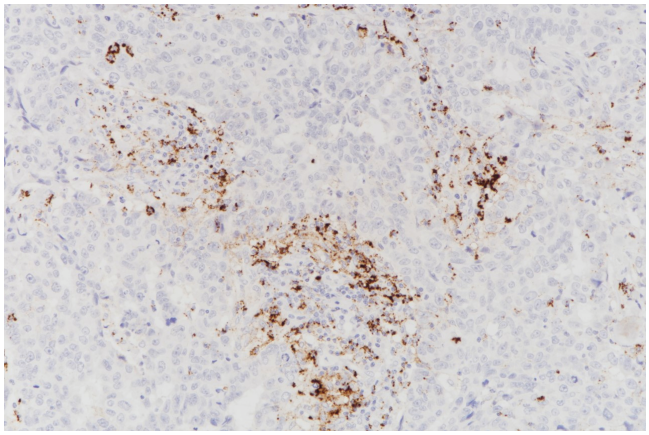

Figure 1. Representative staining of PD-L1-IC (200×).

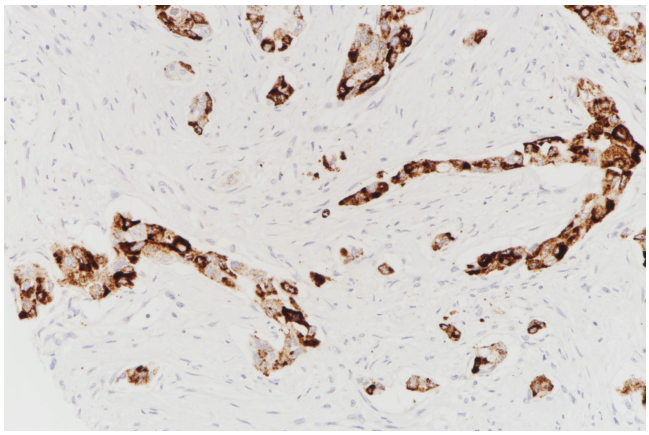

Figure 2. Representative staining of PD-L1-TC (200×).

Table 1. Correlation of PD-L1-IC with clinicopathological features.

		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Posotive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
Grade	1	207	25	232	<.001	-	-	-	1	0	.456	7	0	<.001
	2	551	176	727		-	-		17	12		33	14	
	3	394	399	793		-	-		67	66		79	126	
LVI	Absent	835	413	1248	.115	83	94	.210	57	51	.309	89	114	.365
	Present	271	161	432		61	51		21	24		25	24	
Apocrine	Absent	1029	511	1540	.005	128	130	.739	51	34	.113	96	114	.782
	Present	115	87	202		24	27		56	22		23	25	
FF	Absent	823	459	1282	.016	90	121	<.001	63	67	.072	80	101	.390
	Present	317	133	450		61	31		20	10		39	39	
EIC	Absent	870	496	1366	.001	122	132	.176	61	59	.855	96	130	.064
	Present	248	92	340		26	18		21	19		16	10	
Necrosis	Absent	934	397	1331	<.001	108	102	.396	47	38	.403	71	65	.005
	Present	190	192	382		41	50		36	38		46	75	
sTIL	Low	934	397	1331	<.001	103	40	<.001	46	9	<.001	80	43	<.001
	High	185	190	375		26	88		18	53		24	76	
Pt stage	1	524	218	742	.044	41	47	.332	27	27	.208	43	41	.630
	2	528	333	861		91	97		48	45		58	79	
	3	60	30	90		13	7		6	3		11	12	
	4	30	10	40		3	2		4	1		6	6	
pN stage	0	578	274	852	.019	51	66	.299	36	35	.450	65	75	.616
	1	315	173	488		47	41		24	21		31	38	
	2	129	77	206		29	21		9	16		11	10	
	3	84	58	142		21	23		15	6		8	14	
Molecularsubtype	Lum A	551	132	683	<.001	-	-		-	-		-	-	.002
	Lum B	382	246	628		-	-		-	-		-	-	
	Lum B HER2 pos	69	74	143		-	-		-	-		-	-	
	Lum B HER2 neg	313	172	485		-	-		-	-		-	-	
	Lum B G3 HER2 pos	35	53	88		-	-		-	-		-	-	
	Lum B G3 neg	118	104	222		-	-		-	-		-	-	
	HER2-OE	85	78	163		-	-		-	-		-	-	
	(TNBC)	(119)	(140)	(259)										
	BLBC	39	72	111		-	-		-	-		39	72	
	5NP	80	68	148		-	-		-	-		80	68	
Age	Mean	54.6	52.9	54.0	.007	50.8	51.64	.880	52.1	52.6	.608	56.8	55.5	.393
	SD	12.8	12.4	12.7		11.0	12.27		12.7	11.3		13.9	13.7	.
	Median	52	51	52		51	49.0		51	52		54	53	

Table 1. Continued

		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Posotive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
	Range	27-101	22-94			28-81	22-85		31-87	23-87		28-101	30-94	
Tumor size	Mean	2.59	2.75	2.65	<.001	3.05	2.74	.307	2.98	2.94	.969	2.99	3.01	.366
	SD	1.51	1.37	1.47		1.69	1.61		1.52	1.32		1.75	1.44	
	Median	2.2	2.5	2.30		2.5	2.15		2.5	2.6		2.5	2.8	
	Range	0.1-13.0	0.4-2.6			0.4-13.0	0.7-11 0		0.1-8.0	1.2-7.5		0.5-8.0	0.5-7.6	
ER	Negative	255	260	515	<.001	20	28	.247	85	78	-	118	138	.
	Positive	889	336	1225		133	129							
PR	Negative	307	259	566	<.001	18	16	.672	85	77	-	118	139	-
	Positive	835	336	1171		135	140							
Ki67	Low	978	435	1413	<.001	46	36	.144	38	35	.983	63	56	.030
	High	167	162	329		106	121		47	43		54	83	
HER2	Negative	978	435	1413	<.001	118	104	.034	-	-	-	118	139	-
	Positive	167	162	329		35	53		85	78				
EGFR	Negative	1107	551	1658	<.001	149	145	.110	79	67	.142	107	123	.349
	Positive	27	46	73		4	11		6	11		10	17	
C-kit	Negative	1016	493	1509	<.001	138	134	.182	77	67	.351	94	93	.018
	Positive	115	105	220		14	22		8	11		24	47	
P63	Negative	1098	554	1652	<.001	146	144	.184	76	73	.255	108	129	.725
	Positive	36	42	78		7	13		9	4		7	10	
CK5/6	Negative	1024	484	1508	<.001	145	134	.008	65	66	.175	83	74	.004
	Positive	112	112	224		8	22		18	27		35	66	
CK14	Negative	1092	546	1638	<.001	149	144	.031	84	77	1.00	96	107	.336
	Positive	46	52	98		3	12		1	1		22	33	
Vimentin	Negative	613	298	911	<.001	78	72	.250	48	47	.527	53	43	.003
	Positive	72	71	143		9	14		4	7		14	34	
P-cadherin	Negative	564	242	806	<.001	65	57	.218	20	24	.587	33	29	.138
	Positive	116	124	240		21	28		31	30		33	48	
AR	Negative	341	207	548	.038	47	47	.934	40	37	.523	51	63	.318
	Positive	346	160	506		40	39		14	17		17	14	
PDL1-T	Negative	1016	517	1533	.011	131	137	.779	67	61	.829	106	117	.185
	Positive	98	75	173		20	19		16	17		10	19	
PD1 TIL	Negative	677	316	993	<.001	86	72	.001	53	46	.031	68	66	.001
	Positive	13	52	65		2	16		1	7		0	11	
HVEM	Negative	563	276	839	.001	70	60	.002	25	26	1.00	50	56	.970
	Positive	48	49	97		1	12		20	19		10	11	
CX3CL1	Low	262	133	395	.372	24	34	.118	21	21	.713	25	21	.281

Table 1. Continued

		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Posotive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
	High	186	109	295		28	20		9	11		18	24	
HLA-A	Low	573	203	776	<.001	63	41	<.001	38	24	.006	56	38	<.001
	High	123	183	306		21	50		16	30		13	47	
HLA-B	Low	532	201	733	<.001	57	46	.036	38	27	.026	46	35	.001
	High	178	192	370		30	46		17	29		22	50	
HLA-C	Low	532	201	733	<.001	63	34	<.001	35	21	.008	44	28	<.001
	High	178	192	370		23	57		21	35		24	56	
HLA status	All low	378	115	493	<.001	46	22	<.001	29	15	.041	34	20	<.001
	Mixed	205	147	352		23	39		15	21		23	26	
	All high	68	116	184		11	28		9	18		9	37	

LVI, lymphovascular invasion; FF, fibrotic focus; EIC, extensive intraductal carcinoma; sTIL, stromal tumor-infiltrating lymphocytes.

basal-like breast cancers, the expression of ki67, c-kit, Ck5/6, vimentin, HLA-A, HLA-B, HLA-C, combined HLAs expression status and PD1+TIL ($P \leq .030$), while PD-L1-TC was associated positively with high sTIL, p63 and HLA-A expression ($P \leq .042$) (Tables 1 and 2).

Relationship of PD-L1-IC Expression and PD-L1-TC Expression with Patient's Outcome

Follow-up data were available in 1537 patients with a mean follow-up duration of 73 months (range 1-210 months). Of these, 263 (17.1%) had breast cancer-specific mortality or relapse. High sTIL, PD-L1-IC, and PD-L1-TC expression showed no association with DFS or BCSS in the whole cohort (data not shown). When stratified into molecular subtypes, better DFS and BCSS by high sTIL were shown in both TNBC (DFS: log-rank = 7.691, $P = .006$; BCSS: log-rank = 3.964, $P = .046$) and HER2-OE (DFS: log-rank=5.20, $P = .023$; BCSS: log-rank=7.503, $P = .006$). Although high sTIL did not confer a better survival in all luminal B cancers, in the high–grade luminal B subset, a favorable BCSS was found (log-rank=4.422, $P = .035$). On the other hand, better DFS and BCSS were shown by PD-L1-IC expression in HER2-OE (DFS: log-rank = 5.197, $P = .033$; BCSS: log-rank = 4.099, $P = .043$) and high grade luminal B (DFS: log-rank=4.434, $P = .035$; BCSS: log-rank=6.865, $P = .009$), but not TNBC (Fig. 3). The PD-L1-TC expression was not associated with a significant better outcome in all these subsets (data not shown).

Next, we examined the relationship of PD-L1-IC with sTIL in patients' outcomes. In TNBC, compared with sTIL low PD-L1-IC negative cases, sTIL high cases regardless of PD-L1-IC status showed significantly better DFS (sTIL high PD-L1-ICneg: log-rank = 4.066, $P = .044$; sTIL high PD-L1-ICpos: log-rank = 5.315, $P = .021$). There was only a trend of better BCSS for sTIL high PD-L1-IC positive cases than sTIL low PD-L1-IC negative cases (log-rank = 3.361, $P = .067$). In HER2-OE, compared with sTIL low PD-L1-IC negative cases, sTIL high cases regardless of PD-L1-IC status showed significantly better DFS (sTIL high PD-L1-ICneg: log-rank = 5.392, $P = .020$; sTIL high PD-L1-ICpos: log-rank = 5.601, $P = .018$). Interestingly, sTIL low PD-L1-IC positive cases also demonstrated a better DFS (log-rank = 4.598, $P = .032$). Similar observations were also found regarding BCSS in HER2-OE. In the high–grade luminal B cases, sTIL high PD-L1-IC positive cases showed the best BCSS (sTIL low PD-L1-ICneg: log-rank = 7.099, $P = .008$; sTIL high PD-L1-ICneg: log-rank = 4.547, $P = .033$; sTIL low PD-L1-ICpos: log-rank = 3.163, $P = .075$). No differences were found in DFS for high–grade luminal B cases (Fig. 4).

Relationship of sTIL and PD-L1-IC Expression with Other Immune Components

Further evaluation of the correlation of PD-LI-IC and sTIL subgroups with immune components was performed. In the high–grade luminal B cases, significant differences were found in PD1+TIL, HVEM, HLA-A, HLA-B, HLA-C, and combined HLAs status ($P \leq .040$). However, the differences in PD1+TIL and HVEM were due to the differential distribution between sTIL low PD-L1-IC negative and sTIL high PD-L1-IC positive cases. The pairwise comparison demonstrated significantly higher HLA-A, HLA-B, and HLA-C expression as well as all HLAs high status in sTIL high PD-L1-IC positive than sTIL high PD-L1-IC negative cases (Table 3). In the HER2–OE cases, significant differences

Table 2. Correlation of PD-L1-TC with clinicopathological features.

PDL1 TC		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Positive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
Grade	1	209	15	224	.003	-	-	-	1	0	.881	6	1	.614
	2	644	58	702		-	-		23	6		38	7	
	3	685	100	785		-	-		105	27		179	21	
LVI	Absent	1088	125	1213	.257	148	26	.134	84	24	.110	177	21	.406
	Present	392	36	428		102	10		40	5		40	7	
Apocrine	Absent	1370	131	1501	<.001	228	27	.011	86	19	.329	185	20	.118
	Present	160	41	201		39	12		43	14		37	9	
FF	Absent	1123	126	1249	.829	181	28	.564	108	22	.184	156	22	.511
	Present	399	43	442		81	10		21	8		67	7	
EIC	Absent	1208	131	1339	.777	221	31	.901	95	24	.665	193	26	.748
	Present	296	34	330		38	5		34	7		24	2	
Necrosis	Absent	1186	110	1296	<.001	182	27	.059	71	13	.162	120	13	.613
	Present	317	59	376		78	11		56	18		101	16	
sTIL	Low	951	84	1035	.001	123	19	.487	43	12	.652	110	9	.015
	High	353	57	410		102	12		57	13		79	18	
Pt stage	1	652	66	718	.325	82	8	.832	42	12	.500	75	8	.764
	2	757	92	849		156	30		76	17		117	16	
	3	78	10	88		19	1		6	2		19	4	
	4	34	4	38		5	0		4	1		10	1	
pN stage	0	736	92	828	.061	102	14	.594	54	9	.144	119	18	.247
	1	428	48	476		75	11		23	3		58	9	
	2	186	19	205		42	8		8	0		21	0	
	3	131	8	139		41	3		7	0		20	1	
Molecular subtype	Lum A	626	42	668	<.001	-	-	-	-	-	-	-	-	.900
	Lum B	547	68	615		-	-		-	-		-	-	
	HER2-OE	129	33	162		-	-		-	-		-	-	
	(TNBC)	(223)	(29)	(252)										
	BLBC	95	12	107		-	-		-	-		95	12	
	5NP	128	17	145		-	-		-	-		128	17	
Age	Mean	54.1	54.1	54.1	.899	51.1	452.23	.409	52.1	53.3	.840	56.2	55.4	.655
	SD	12.7	13.4	12.8		11.6	10.8		11.9	12.6		13.8	15.0	
	Median	52	52	52		50	52		52	52		54	52	
	Range	22-101	30-89			22-85	31-78		23-87	35-80		28-101	30-89	
Tumor size	Mean	2.65	2.72	2.66	.870	2.95	2.69	.506	2.96	2.89	.697	2.98	3.17	.844
	SD	1.45	1.59	1.47		1.54	1.03		1.42	1.46		1.54	1.88	
	Median	2.3	2.4	2.3		2.5	2.4		2.5	2.7		2.6	2.5	

Table 2. Continued

PDL1 TC		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Positive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
	Range	0.1-13.0	0.5-11.0			0.4-13.0	1.2-6.2		0.1-7.5	1.2-8.0		0.5-8.0	0.5-8.0	
Biomarkers														
ER	Negative	430	75	505	<.001	40	7	.624	129	33		225	32	-
	Positive	1099	98	1197		228	32					-	-	
PR	Negative	484	69	553	.026	29	3	.546	128	33		227	32	-
	Positive	1042	103	1145		238	36					-	-	
Ki67	Low	1002	104	1106	<.001	70	11	.793	64	9	.021	103	10	.210
	High	559	100	659		197	28		65	24		117	19	
HER2	Negative	1260	117	1377	<.001	196	24	.133	-	-	-	221	29	-
	Positive	272	55	327		72	15		129	33		-	-	
EGFR	Negative	1464	155	1619	.001	256	35	.109	121	24	<.001	196	27	.750
	Positive	57	16	73		11	4		8	9		25	2	
C-kit	Negative	1464	155	1619	.950	239	23	.171	115	28	.493	159	23	.383
	Positive	57	16	73		28	4		14	5		63	6	
P63	Negative	1459	156	1615	.002	262	35	.311	119	29	.339	208	22	.001
	Positive	61	16	77		16	4		9	4		11	6	
CK5/6	Negative	1334	143	1477	.060	241	36	1.00	105	25	.364	136	18	.933
	Positive	187	30	217		26	3		22	8		86	11	
CK14	Negative	1435	168	1603	.102	252	39	.232	127	33	1.00	171	26	.151
	Positive	90	5	95		15	0		2	0		51	3	
Vimentin	Negative	829	76	905	.829	133	16	.686	79	15	.396	86	9	1.00
	Positive	129	11	140		19	3		8	3		43	4	
P-cadherin	Negative	748	52	800	<.001	112	9	.032	37	7	.747	55	5	.754
	Positive	205	33	238		39	9		49	11		73	8	
AR	Negative	494	50	544	.206	80	13	.193	63	13	.903	103	10	.846
	Positive	462	25	497		72	6		26	5		27	3	
PD1 TIL	Negative	901	75	976	.152	141	16	.297	83	15	.612	120	12	1.00
	Positive	55	8	63		13	3		6	2		10	1	
HVEM	Negative	769	62	831	.042	113	15	.658	40	10	.369	96	8	.107
	Positive	84	13	97		11	2		34	5		17	4	
CX3CL1	Low	361	31	392	.168	51	5	.143	33	8	.623	40	6	.743
	High	259	32	291		39	9		15	5		37	4	
HLA-A	Low	709	61	770	.011	91	12	.964	50	12	.934	87	5	.042
	High	263	39	302		62	8		38	9		50	9	
HLA-B	Low	661	64	725	.576	88	13	.465	52	13	.771	73	7	.793
	High	329	36	365		68	7		37	8		63	7	

Table 2. Continued

PDL1 TC		Overall				LumB G3			HER2-OE			TNBC		
		Negative	Positive	Total	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value	Negative	Positive	*P*-value
HLA-C	Low	591	51	642	.054	85	10	.661	45	11	.844	68	4	.162
	High	379	49	428		69	10		45	10		67	10	
HLA status	All low	449	43	492	.139	58	9	.641	35	9	.815	51	3	.406
	Mixed	315	32	347		53	8		30	6		42	5	
	All high	157	25	182		36	3		20	6		39	6	

LVI, lymphovascular invasion; FF, fibrotic focus; EIC, extensive intraductal carcinoma; sTIL, stromal tumor-infiltrating lymphocytes.

were found in HLA-A and combined HLAs status ($P \leq .027$). The pairwise analysis demonstrated that significantly higher HLA-A, HLA-B, and HLA-C expression as well as all HLAs high status in sTIL high PD-L1-IC positive than sTIL low PD-L1-IC negative cases. sTIL high PD-L1-IC positive cases also showed significantly higher HLA-A, HLA-C as well as all HLAs high status than sTIL high PD-L1-IC negative cases (Table 3). In the TNBC, similar to the other 2 subgroups, significant differences were found in the HLAs expression and status ($P \leq .031$). Additionally, significant differences were found in grade ($P < .001$) and TNBC subtypes ($P = .045$). For the HLAs, the differences were mainly due to their higher levels of high PD-L1-IC positive cases than sTIL low PD-L1-IC negative cases or with sTIL high PD-L1-IC negative cases (Table 3).

Discussion

Assessment of PD-L1 expression by IHC in TCs and/or ICs has been used as a clinical biomarker to select patients for treatment with PD-1/PD-L1 inhibitors. The combination of atezolizumab and nab-paclitaxel chemotherapy has been approved for the first-line treatment of patients with locally advanced or metastatic TNBC initially and the SP142 Ventana test has been approved as its complementary diagnostics,[8] but this approval has recently been withdrawn due to the failure of subsequent study IMpassion131 in meeting its primary endpoint for the treatment of people with mTNBC in the PD-L1-positive population. This discrepancy suggests the insufficiency of prognostication of PD-L1 along in breast cancer, and other factors need to be combined in prognosis prediction and immunotherapy regimen making.

Apart from SP142, multiple assays, such as Ventana SP263 for durvalumab, DAKO 22C3 for pembrolizumab; and DAKO 28-8 for nivolumab, have been developed for PD-L1 assessment with variable scoring criteria and staining protocol.[22] However, the SP142 assay appeared to generate different results, with the least concordance with the other assays[23,24] and showing a lower sensitivity.[25] Also, differing from the others, the assessment of SP142 was based mainly on IC, rather than TC. Inter-observer variability could be high for its assessment.[26] Earlier studies on PD-L1 expression mainly focused on TC and using other antibody clones.[18,27,28] A few recent analyses on IC using SP142 assays examined only TNBC cases.[11-14] Given immunotherapy can be exploited not only in TNBC but also other subtypes, here, PD-L1 expression was evaluated with PD-L1 antibody clone SP142 on both TC and IC, with further correlation analysis performed with clinical and histological features, biomarkers expression, and molecular subtypes.

In our cohort, the overall PD-L1-IC expression rate in breast cancer is 34%, with a much lower PD-L1-TC expression rate of 10.1%. These findings were similar to the previous studies showing the preferential staining of SP142 on IC in cancers from other tissues.[29] In TNBC, PD-L1-IC expression by SP142 was reported from 28 to 56% while a range of 5-37% was reported for its TC expression.[12,13,23,30] In our cohort, the PD-L1 expression rates on TNBC IC and TC were 57.5% and 11.6%, respectively, similar to those reported previously. These figures were close to the upper boundary of the reported range, higher than that in the IMpassion130 trial.[8] It could be due to the fact that only primary TNBCs were included in the current analysis while both primary and

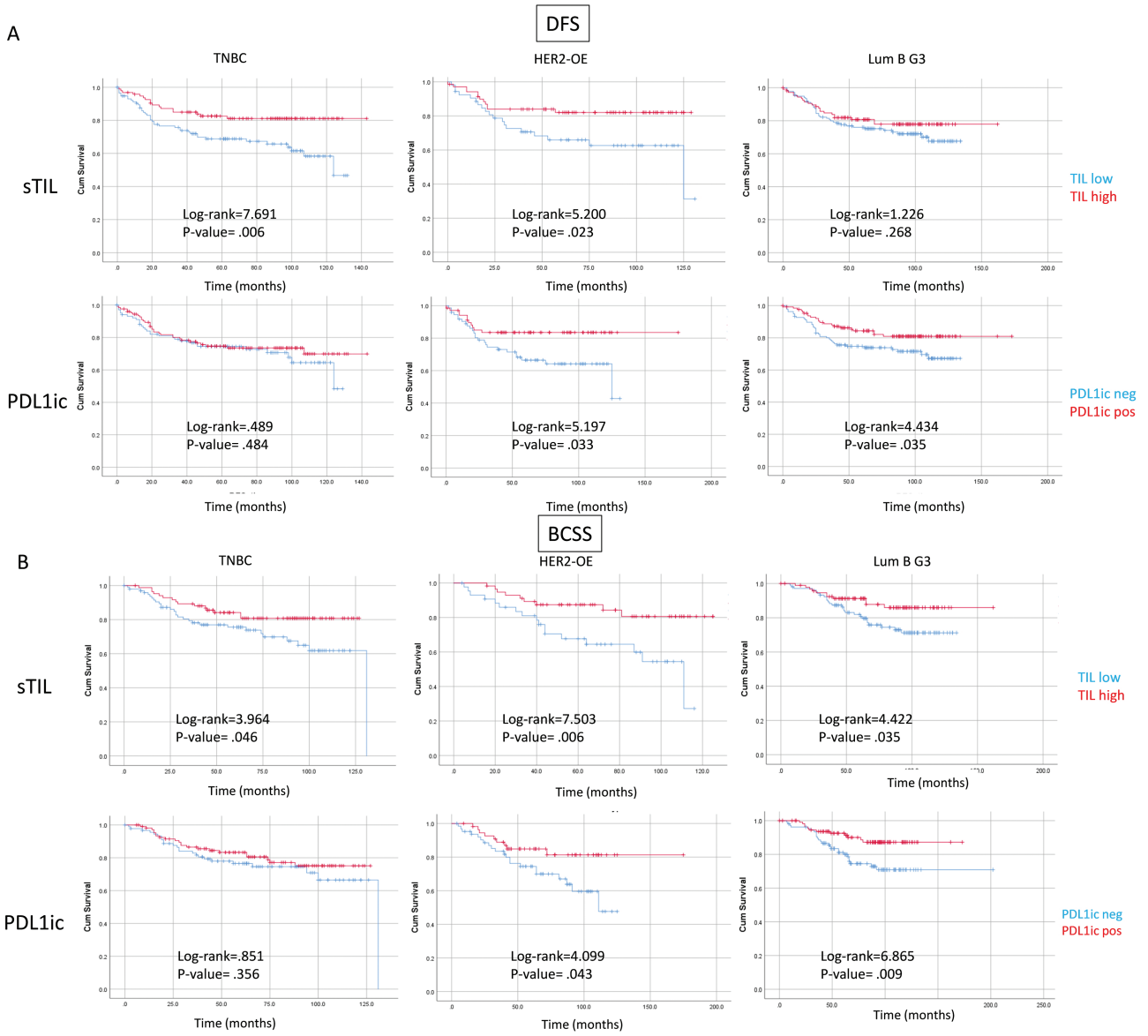

Figure 3. Kaplan–Meier analysis of DFS (**A**) and BCSS (**B**) according to sTIL and PD-L1-IC in TNBC, HER2-OE, and Luminal B G3 cases.

metastatic TNBCs were included in the trial. Metastatic cancers may have lower TILs and PD-L1 expression.[31] We found positive associations between PD-L1-IC expression and unfavorable prognostic factors, namely higher tumor grade, tumor necrosis, larger tumor size, and lymph node metastases. There was no association in TNBC with tumor size and lymph node metastasis, which is also in line with a recent study.[15] By contrast, positive associations with grade, higher TIL, PD1+TIL, higher expression of HLAs, BLBC subtype by IHC surrogate, and markers showed high expression in BLBC (namely ki67, CK5/6, vimentin, and c-kit) were observed. Given that SP142 was more sensitive to IC, its association of higher TIL and PD1+TIL could be expected. It has been shown that approximately 20% of TNBC classified as immunomodulatory subtype which was highly enriched in immune cell markers and signaling was fell into PAM50 BLBC.[32] PD-L1-IC positive cases would be enriched with the immunomodulatory TNBC. This could account for its association with the BLBC subtype by IHC surrogate.

High TIL, but not PD-L1-IC positivity, was related to better survival in our TNBC cohort. In addition to PD-L1, IFNγ activated other IFNγ signature genes, including HLA, to mediate active immunity.[33] It appeared that, compared to PD-LI-IC negative low TIL cases, only cases with PD-L1-IC positive high TIL demonstrated a higher level of all HLA-A, HLA-B, and HLA-C as well as their co-expression status. HLA upregulation and high TIL may represent an active IFNγ response, thus an effective anti-tumor immunity. PD-L1-IC expression alone without commitment high TIL and HLA may represent a compromised anti-tumor immunity. In line, in our previous studies, significantly better survival was found in only cases with all HLA high and high TIL in a subset of breast cancers.[20] In melanoma patients treated with ICB, HLA status was related to survival, independent of somatic mutational load, tumor stage, age, or types of treatment.[34] Interestingly, in the phase II GeparNuevo trial for TNBC, HLA-A, and HLA-B were among the 44 genes significantly correlated with pCR in the durvalumab treatment arm.[35] Moreover, a

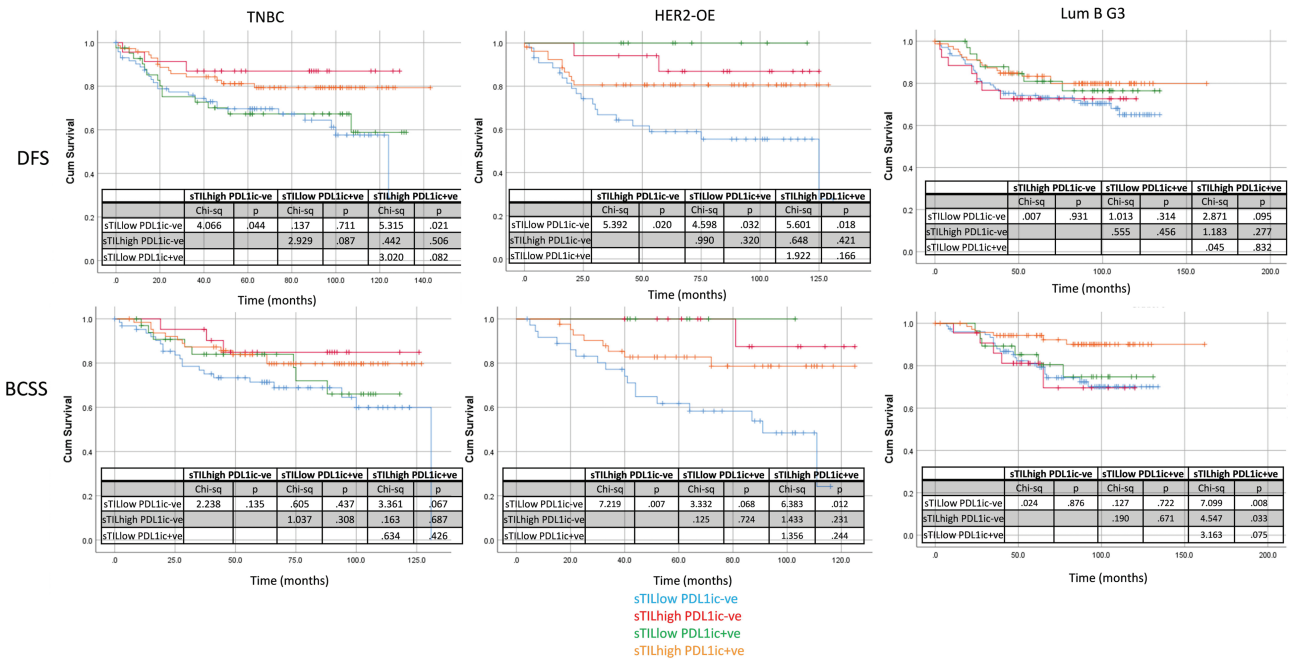

Figure 4. Kaplan–Meier analysis of DFS and BCSS according to sTIL and PD-LI-IC combination in TNBC, HER2-OE, and Luminal B G3 cases.

high TIL level has been associated with a greater chance of achieving response to pembrolizumab monotherapy in phase 2 KEYNOTE-086 study of previously treated mTNBC.[36] Regarding ICB in breast cancer, other immune markers, such as high TIL and HLA, together with PD-L1 expression could be also useful in refining the prediction of treatment response.

Apart from TNBC, a remarkable level of PD-L1-IC expression rate can be found also in HER2-OE (47.9%) and luminal B (39.2%), but the least was found in luminal A (19.3%). For PD-L1-TC, its expression in HER2-OE (20.3%) was the highest, similar rates were found for TNBC (11.5%) and luminal B (11.0%) and the least in luminal A (6.2%). These results are parallel to the observation that reported previously on high–grade breast cancer in general,[37] implicating the potential validity of ICB in other subtypes of breast cancers. For HER2–positive cancers, high TIL, as shown by us and others have been associated with a better prognosis independent of other clinicopathological characteristics.[18] Immune-mediated mechanisms have been shown, at least partly, to contribute to the treatment outcome of the standard treatment of HER2–positive cancers including both anti-HER2 therapy and chemotherapy.[38] It has implicated in a synergistic action of ICB with these standard treatments. It is interesting to note that survival benefit was found in PD-LI-IC positivity and/or high TIL in our HER2-OE cases. The results echoed with data from KATE2, a randomized phase 2 study that evaluated atezolizumab with trastuzumab emtansine (T-DM1) in previously treated HER2+ advanced breast cancer. Numerically higher progression-free survival and overall response rate with atezolizumab and T-DM1 treatment in PD-L1+/high TIL patients, despite limited data, have been presented so far.[39] It is also intriguing to observe that better DFS of sTIL low PD-L1-IC positive cases in HER2-OE cancer. This subset represented a very small proportion (7.1%) in our HER2–OE population. With the small case number, further validation of their association with better survival is warranted. Nonetheless, the results echoed the data from the KATE2 trial evaluating atezolizumab with T-DM1 as mentioned above. For HER2–OE breast cancer, targeted treatment with a humanized antibody could be applied. The predominant immune cell type that expresses PD-L1 is macrophage in the tumor microenvironment.[40] It is possible that the presence of these PD-L1 IC, presumably macrophages, interact with the Fc portion of the therapeutic antibody via its FcgRs, leading to antibody-dependent cellular phagocytosis.[41] Even with the low basal TIL level, the killing of cancer cells by antibody-dependent cellular phagocytosis could further stimulate the downstream immune response. Interestingly, the anti–HER2 antibody could also interact with NK cells directly, resulting in upregulation of HLA and IFNg secretion.[42] These may at least partly explain the differences in those cases with sTIL low PD-L1-IC positive among the HER2–OE breast cancer.

Luminal breast cancers have been considered a "cold" tumor with a low level of TIL. Immunotherapy in these breast cancers is underexplored. However, this subtype is highly heterogeneous and some subset exhibits elevated TILS levels. In fact, some recent clinical trials implicated the potential of immunotherapy in luminal cancers. In the phase II GIADA trial, a pCR rate of 16% was reported in premenopausal luminal B cancers upon sequential anthracycline chemotherapy and nivolumab treatment.[43] In our high–grade luminal B cases, they demonstrated high TIL, PD1+TIL, and HLAs expression as well as benefit in BCSS for high TIL/PD-L1-IC as in HER2-OE. Notably, mainly those high TIL cases with PD-L1-IC positivity showed a better BCSS. There could be immunological differences among different breast cancer subtypes, despite the high TIL infiltration. A recent study has shown that immune-rich ER+ cancers have shown to express TGF-β response metagenes and enrich with M2-like macrophage gene signature.[44] Of note, high TGF-β signaling has been associated with lesser response to immune checkpoint inhibitors.[45] Despite the expression of PD-L1, for optimal ICB treatment in other breast cancer

Table 3. Correlation of sTIL-PD-L1-IC combinations with immune features.

		LumB				HER2-OE				TNBC			
		sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve	sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve	sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve
PD1 TIL	Negative	45[a]	17	16	34[a]	22	11	5	27	37[a]	16	17	33[a]
	Positive	1	1	1	8	1	0	1	4	0	0	1	5
	P-value	.040				.418				.061			
CX3CL1	Low	16	6	6	19	11	6	4	15	17	4	4	13
	High	14	9	5	10	5	0	1	6	9	7	7	13
	P-value	.441				.468				.256			
HVEM	Negative	45	17	16	32	10	6	3	19	31	14	16	31
	pos	0	1	1	9	13	5	4	12	7	2	2	7
	P-value	.004				.575				.856			
HLA-A	Low	32[a]	15[b]	9	18[a,b]	17[a]	8[b]	4	11[a,b]	34[a]	12[b]	12	18[a,b]
	High	13	2	8	29	6	4	3	23	7	3	8	26
	P-value	.001				.013				<.001			
HLA-B	Low	29[a]	13[b]	10	18[a,b]	17[a]	7	4	15[a]	28[a]	11[b]	11	19[a,b]
	High	16	5	7	29	6	6	3	19	13	4	9	25
	P-value	.027				.174				.065			
HLA-C	Low	11	7[b]	3	9[b]	11[a]	8[b]	3	10[a,b]	29[a,c]	7[c]	10	14[a]
	High	8	5	2	23	13	5	4	24	11	9	10	30
	P-value	<.001				.134				<.001			
HLA-status	All low	25[a]	11[b]	5	7[a,b]	12[a]	6[b]	3	5[a,b]	22[a]	6[b]	8	10[a,b]
	Mixed	9	4	8	22	7	2	1	17	13	6	5	14
	All high	9	1	4	16	3	4	3	12	5	3	7	19
	P-value	<.001				.027				.031			
Grade	1	-	-	-	-	1	0	0	0	4[a,c]	2[b]	0[c]	0[a,b]
	2	-	-	-	-	13	1	2	5	23	5	5	5
	3	-	-	-	-	32	17	7	48	53	17	38	71
	P-value	-				.113				<.001			
Subtypes	BLBC	-	-	-	-	-	-	-	-	29[a]	7[b]	18	42[a,b]
	5NP	-	-	-	-	-	-	-	-	51	17	25	34
	P-value	-				-				.045			
LVI	No	62	13	26	52	33	14	4	39	61	18	36	62
	Yes	38	12	13	35	11	4	5	14	15	6	7	13
	P-value	.688				.264				.818			
Node metastasis	No	34	9	16	37	21	10	5	24	39	15	21	40
	Yes	64	17	21	49	23	8	4	29	36	8	22	43

Table 3. Continued

	LumB				HER2-OE				TNBC			
	sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve	sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve	sTIL low PDL1ic-ve	sTIL high PDL1ic-ve	sTIL low PDL1ic+ve	sTIL high PDL1ic+ve
P-value	.606				.857				.624			

[a]Pairwise comparison between sTIL low PD-L1-IC negative and sTIL high PD-L1-IC positive cases.
[b]Pairwise comparison between sTIL high PD-L1-IC negative and sTIL low PD-L1-IC positive cases.
[c]Pairwise comparison between sTIL low PD-L1-IC negative and sTIL high PD-L1-IC negative cases.
LVI, lymphovascular invasion.

subtypes, different strategies and/ or patient selection should be considered.

A limitation of this study was the use of TMA for PD-L1 assessment. Despite duplicated cores being used, there could be potential differences in results from TMA and the whole section. However, several reports have shown comparable results obtained from small biopsies/TMA with whole sections.[46,47] Therefore, TMA can serve as an affordable alternative in a research setting. Although the study included a large cohort of breast cancer, there were only limited cases in some subset analysis for which further validation will be required.

Conclusion

Our study included the largest cohort in investigating the PD-L1 expression by SP142 in breast cancer, on both TC and IC. PD-L1 expression is much more prevalent in IC than TC. The highest PD-L1-IC expression was found in the TNBC subtype. A remarkable rate of approximately 50% was also observed in HER2-OE and high–grade luminal B. Despite the association with high TIL level, PD-L1-IC positivity did not demonstrate a favorable survival in TNBC. Combining analysis of TIL and PD-L1-IC refine the prognostication of breast cancer subtypes. Cases with high TIL and PD-LI-IC appear to be more immune active.

Conflict of Interest

The authors indicated no financial relationship with any companies.

Author Contributions

Conception/design: Y.N., and G.T. Provision of study material or patients: Y.N., F.T., K.-H. S., and G.T. Collection and/or assembly of data: Y.S., and I.K.P. Data analysis and interpretation: Y.N., and J.Y.T. Manuscript writing: Y.N., J.Y.T., and G.T. Final approval of manuscript: All authors.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Supplementary Material

Supplementary material is available at *The Oncologist* online.

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