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The Expression Level of *PD-L1* (*CD274*) mRNA in Peripheral Blood Is a Potential Biomarker for Predicting Recurrence in Breast Cancer

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Abstract. *Background/Aim: Programmed death-ligand 1 (PD-L1/CD274) elicits T-cell anergy, leading to immune suppression. We aimed to determine the prognostic relevance of PD-L1 expression in the blood of breast cancer (BC) patients. Materials and Methods: We measured PD-L1 mRNA expression in blood and tumor tissues of BC patients using RT-qPCR and a dataset from The Cancer Genome Atlas, and performed a survival analysis of PD-L1 expression in the blood of 330 BC patients. Flow cytometric analysis was performed using blood cells. Results: No statistical difference in PD-L1 expression was seen between normal controls and BC in blood or tissues. There was a significant positive correlation between the PD-L1 expression levels in blood and tissues. Decreased PD-L1 expression in blood or tissues was associated with poor recurrence-free survival. PD-L1 is mainly expressed in polymorphonuclear leukocytes. Conclusion: Low expression of PD-L1 in the blood could serve as a biomarker of poor prognosis in BC patients.*

The immune system plays an essential role in the surveillance and eradication of neoplastic cells. In fact, cancer immunotherapy has become the fourth pillar of cancer treatment, complementing surgery, chemotherapy and radiotherapy (1). In cancer immunotherapy, development of immune checkpoint inhibitors (ICI), which block the immunosuppressive effects of tumors on T cells, have become considerably important in the clinic (2, 3).

Programmed death-ligand 1 (PD-L1/CD274) is a transmembrane protein that binds to the inhibitory receptor PD-1 (PDCD1) on T cells where it elicits T-cell anergy, leading to immune suppression (4). Many cancer cells upregulate PD-L1 surface expression to escape immunosurveillance (5, 6). ICI, including neutralizing antibodies targeting PD-L1 or PD-1, can block T-cell anergy and thereby resensitize tumor cells to antitumor immunity (6). This approach has shown significant therapeutic promise in the treatment of various cancers (7, 8), such as PD-L1-positive metastatic triple-negative breast cancer (TNBC) combined with anti-cancer drug nab-paclitaxel (9-12).

Among multiple cancer types, positive responses to PD-1/PD-L1-targeted therapy have been observed in patients with high PD-L1 expression on tumors cells, as revealed by immunohistochemical staining (tumor proportion score ≥1%) (13). However, these biomarkers require invasive sampling and are not practical from a risk-benefit standpoint for monitoring tumor responsiveness during treatment. Furthermore, many studies investigating the prognostic implications of PD-L1 expression in tumor tissues of breast cancer (BC) patients have reported conflicting results, probably because of tumor heterogeneity (14).

There is growing interest in the use of "liquid biopsies" to identify biomarkers in body fluids, mainly in the blood (15). Circulating liquid biopsy biomarkers have recently shown promise as a metric for the prediction of tumor immunotherapy responses. This approach is less invasive and more cost-effective in providing "real-time" information regarding not only tumor characteristics but also the immune status of cancer patients (15-17). In this context, we have

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reported that expression of immune-related genes including *PD-1* mRNA in the blood could be a promising prognostic factor in primary gastric cancer or BC (18, 19). However, the clinical significance of *PD-L1* mRNA expression in the blood remains unclear in BC, although the prognostic significance of serum PD-L1 protein level has been reported in several malignancies (20).

In this study, we aimed to determine the clinicopathological and prognostic significance of *PD-L1* mRNA expression in the blood of BC patients who underwent curative surgery.

Materials and Methods

Clinical samples. To determine the clinical significance of PD-L1 expression in the blood, we used clinical data files from BC patients as previously described (21, 22). Briefly, a total of 594 patients with BC underwent resection of the primary tumor at the Department of Breast Oncology, National Kyushu Cancer Center (Fukuoka, Japan) from 2000 to 2008. This cohort included 356 female patients with breast cancer without distant metastases, who underwent curative surgery, preoperative therapy or previous treatment for various cancers. Among them, 330 were diagnosed with invasive ductal carcinoma (IDC), and 26 with ductal carcinoma *in situ* (DCIS). Survival analysis was performed for the 330 patients with IDC. The control samples were obtained from 11 healthy volunteers (HVs) at Kyushu University Beppu Hospital. Aspiration of blood was conducted immediately before surgery under general anesthesia. Blood was obtained through a venous catheter. The first 1 ml of blood was discarded to avoid possible contamination by epidermal cells. Each 1 ml of blood was immediately mixed with 4 ml of ISOGEN-LS (Nippon Gene, Toyama, Japan) and stored at -80˚C until RNA extraction.

Polymorphprep™ (Alere Technologies AS, Oslo, Norway) was used to obtain 2 immune blood cell fractions (polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC)] as described before (19, 21, 22). Samples were obtained from 10 patients with IDC who underwent primary tumor resection at Kyushu University Beppu Hospital in 2017 and 10 HVs. Each fraction was mixed with ISOGEN II (Nippon Gene, Toyama, Japan) and stored at –80˚C until RNA extraction.

Tumor tissues and paired blood samples were obtained from 16 BC patients who had undergone primary tumor resection at Kyushu University Beppu Hospital from 2016 to 2019.

All patients provided written informed consent to participate in this study, which was approved by the Ethics and Indications Committee of Kyushu University (Approval Number: 29-597) and National Kyushu Cancer Center (Approval Number: 2018-31). The observation periods ranged from 0.3 to 6.9 years (median=3.8 years). Postoperative adjuvant therapy was performed according to the St. Gallen Consensus Conference (23). The patients underwent clinical examinations at least every 3 months in addition to annual mammographies. They were further tested only if they had symptoms.

The stages and grades of the tumors were classified according to the AJCC/UICC TNM classification and stage groupings. All data for the samples, including age, pathological tumor size, nuclear grade, venous involvement, lymphatic involvement, lymph node metastasis, and the status of estrogen receptor (ER), progesterone receptor (PgR) and human epidermal growth factor receptor 2 (HER2) were obtained from medical records. The status of ER, PgR and HER2 was determined by immunohistochemical analysis. We defined 0/1+ HER2 status as negative and 2+/3+ as positive. Recurrence-free survival (RFS) was defined as the length of time after surgical treatment for cancer during which the patient survived without a sign of recurrence.

Extraction of total RNA. Frozen tissue specimens were homogenized and total RNA was extracted with ISOGEN according to the manufacturer's instructions. Total RNA of blood was extracted according to the ISOGEN-LS manufacturer's instructions (Nippon Gene).

Reverse transcritption-quantitative polymerase chain reaction (RT-qPCR) assay. RT-qPCR assessments of *PD-L1* and *GAPDH* were performed as previously described (24). In brief, RT was performed with random hexamers using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR was performed with LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics). The raw data are presented as the relative quantity of target genes, normalized to *GAPDH*. The primer sequences for RT-PCR were as follows: *PD-L1*, forward 5'- ACGAGGGACAATAGGAGCCAG-3' and reverse 5'-GCCCATTCCGCTAGGAAAGAC-3' and *GAPDH*, forward, 5'-TTGGTATCGTGGAAGGACTC-3' and reverse 5'-AGTAGAGGCAG GGATGATGT-3'.

Relative quantitation of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The raw data are presented as the relative quantity of each cloned plasmid or cDNA from Human Universal Reference Total RNA (Clontech Laboratories) and normalized to the *GAPDH* internal control. The PCR product was electrophoresed through 3% agarose gel and stained with ethidium bromide.

Public datasets. We used TCGA data to analyze the expression of *PD-L1* mRNA in breast cancer tissues. The expression data of these genes in tumor tissues from 1,093 breast cancer cases and in normal tissues from 112 cases with breast cancer available in TCGA were obtained from the Broad Institute's Firehose pipeline. In this group, 307 of 1,093 were TNM Stage I, II or III and had undergone curative surgery. The sequencing data were normalized with quantile normalization (25).

Flow cytometric analysis (FCM). FCM was performed to identify PD-L1-expressing cells in immune cells (PMN and MC) in the blood obtained from 6 BC patients using phycoerythrin (PE) anti-human PD-L1 mouse monoclonal antibody (Cat. No. 2248525, clone 29E.2A3, isotype Mouse IgG2b K, Sony, San Jose, CA, USA) and mouse IgG2 and mouse IgG2b K isotype control Alexa Fluor 488 (Cat. No.53-4732, clone eBMG2b, isotype mouse IgG2b K, Affymetrix, Santa Clara, CA, USA) as the negative control, as previously described (18). The stained cells were analyzed on a cell sorter (SH800, Sony Biotechnology, San Jose, CA, USA) after gating lymphocytes based on forward and side scatter.

Laboratory data collection. Blood neutrophil and lymphocyte counts were collected from 16 BC patients who had undergone primary tumor resection at Kyushu University Beppu Hospital from 2016 to 2019. The neutrophil-to-lymphocyte ratio (NLR) was calculated by dividing the neutrophil count by the lymphocyte count obtained before surgery.

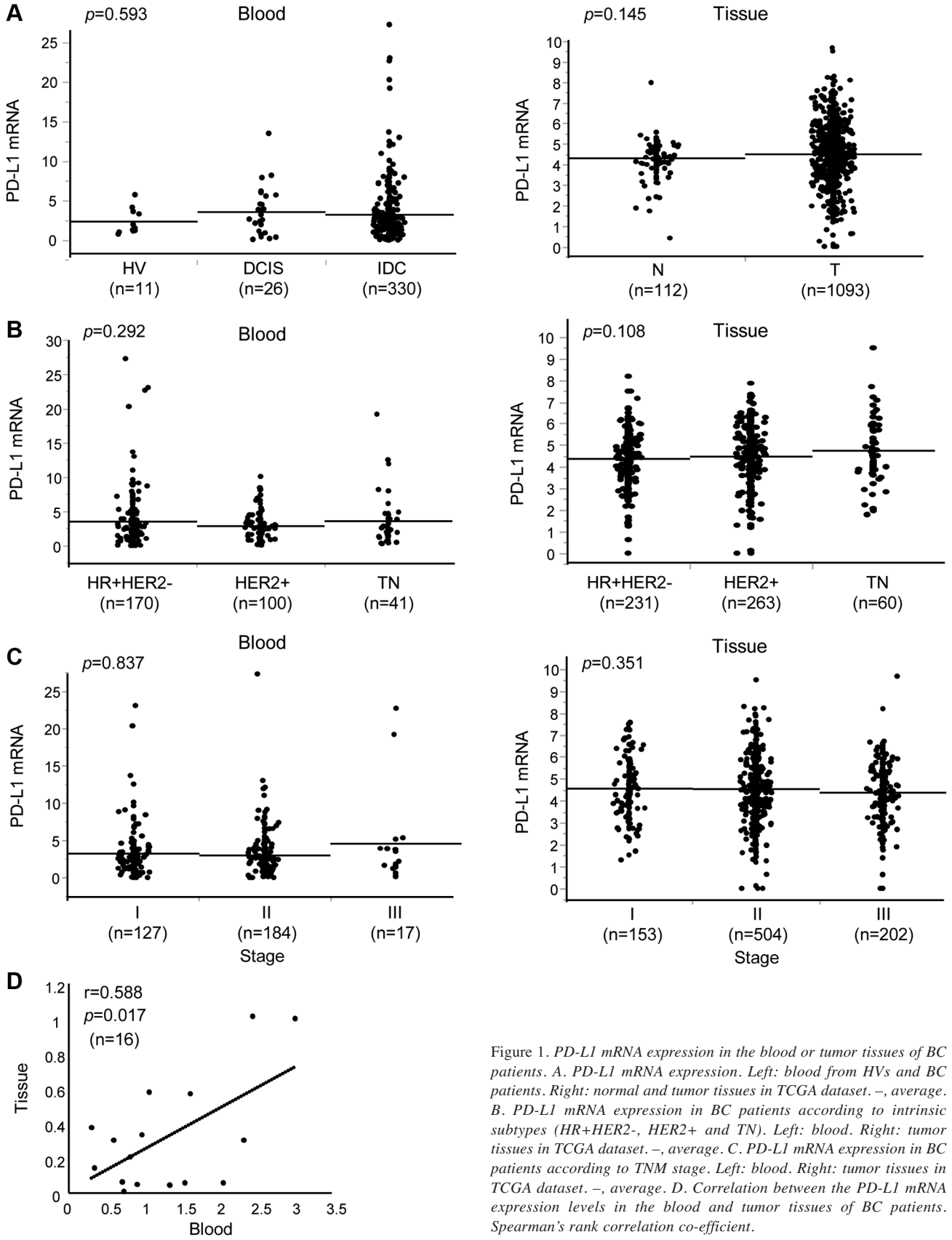

Figure 1. *PD-L1 mRNA expression in the blood or tumor tissues of BC patients. A. PD-L1 mRNA expression. Left: blood from HVs and BC patients. Right: normal and tumor tissues in TCGA dataset. –, average. B. PD-L1 mRNA expression in BC patients according to intrinsic subtypes (HR+HER2-, HER2+ and TN). Left: blood. Right: tumor tissues in TCGA dataset. –, average. C. PD-L1 mRNA expression in BC patients according to TNM stage. Left: blood. Right: tumor tissues in TCGA dataset. –, average. D. Correlation between the PD-L1 mRNA expression levels in the blood and tumor tissues of BC patients. Spearman's rank correlation co-efficient.*

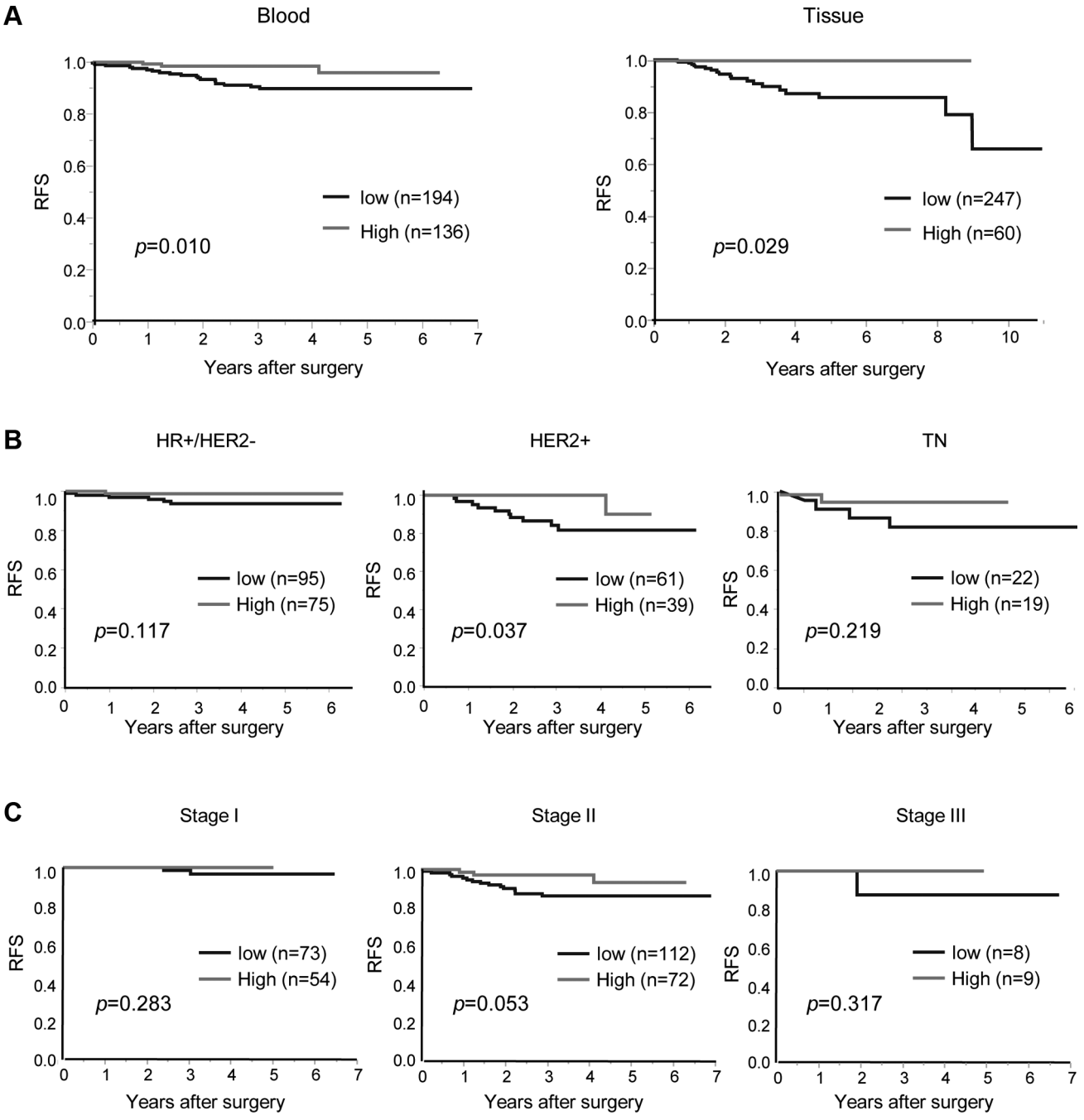

Figure 2. *Kaplan–Meier survival curve of breast cancer patients according to PD-L1 mRNA expression level. A. RFS. left; blood, right; tumor tissues in TCGA dataset. B. Subgroup analysis for RFS of patients according to PD-L1 mRNA expression level in the blood by the intrinsic subtypes (HR+HER2-, HER2+ and TN). C. Subgroup analysis of RFS of patients according to PD-L1 mRNA expression level in the blood by TNM stage (I, II and III). HR: Hormone receptor; TN: triple negative.*

Statistical analysis. For clinical analysis, cases were divided into two groups using the minimum *p*-value approach based on the *PD-L1* mRNA expression level, which is a comprehensive method to identify the optimal risk separation cutoff point in continuous gene expression measurements for survival analysis in multiple datasets (26). Associations between the variables were tested with the Student's t-test or Fisher's exact test. The degree of linearity was estimated by Spearman's rank correlation co-efficient. Survival curves were drawn according to the Kaplan–Meier method, and survival analysis was carried out with the log-rank test when two

Table I. *Univariate and multivariate analyses of prognostic factors for RFS of breast cancer patients.*

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*p*-Value	HR (95%CI)	*p*-Value
Age (y.o) (≥65/<65)	0.58 (0.17-1.96)	0.378		
Pathological tumor size (2, 3/1)	2.34 (0.95-5.73)	0.064	1.69 (0.64-4.81)	0.290
Nuclear grade (3/1, 2)	1.70 (0.72-4.04)	0.228		
Venous involvement (+/–)	1.862e-9 (1.46-1.46)	1.0		
Lymphatic involvement (+/–)	4.68 (1.83-11.96)	0.001		
Lymph node metastasis (+/–)	3.84 (1.56-9.41)	0.003	3.56 (1.37-10.02)	0.009
ER (+/–)	0.48 (0.20-1.11)	0.087		
PgR (+/–)	0.39 (0.16-0.92)	0.032	0.46 (0.17-1.19)	0.110
HER2 (2, 3/0, 1)	2.14 (0.93-4.94)	0.075	1.73 (0.67-4.44)	0.252
PD-L1 mRNA in blood (low/high)	4.30 (1.27-14.56)	0.019	4.73 (1.52-20.80)	0.006

CI: Confidence interval.

curves were being compared. Cox proportional hazards regression was used to determine univariate and multivariate hazard ratios for survival. Variables that achieved statistical significance ($p<0.05$) or those close to significance ($p<0.1$) in the univariate analysis were subsequently included in a multivariate analysis. A two-sided $p<0.05$ was deemed statistically significant. Statistical analyses were performed using JMP Pro 14 software (SAS Institute, Cary, NC, USA).

Results

PD-L1 mRNA expression in the blood or tumor tissues of BC patients. The expression levels of *PD-L1* mRNA in the blood from 330 patients with IDC, 26 patients with DCIS and 11 healthy volunteers (HVs) are plotted in Figure 1A (Left). There were no significant differences in *PD-L1* expression levels in the blood between HVs, and patients with DCIS or IDC. Next, we assessed *PD-L1* mRNA expression in normal and IDC tissues using the TCGA dataset (Figure 1A Right). There was also no significant difference in *PD-L1* mRNA expression levels between them (Figure 1A Right).

Next, we assessed *PD-L1* mRNA expression in the blood or tumor tissues of BC patients categorized by intrinsic subtypes (HR+HER2-, HER2+ and TN). As shown in Figure 1B, there were no significant differences between them in regard to *PD-L1* mRNA expression levels in either blood or tissues.

Finally, we assessed *PD-L1* mRNA expression in the blood or BC tissues according to TNM stage (I, II and III). As shown in Figure 1C, there was no significant difference in *PD-L1* mRNA expression levels in either blood or tissues between TNM stages.

Correlation between the levels of PD-L1 mRNA expression in the blood and tumor tissues of BC patients. We examined the correlation between *PD-L1* mRNA expression levels in the blood and tumor tissues of BC patients in our hospital. Of note, Spearman's rank correlation co-efficient demonstrated a significantly positive correlation between *PD-L1* mRNA expression levels in the blood and tumor tissues (r=0.588, $p=0.017$) (Figure 1D).

Prognostic significance of PD-L1 mRNA expression in the blood or tumor tissues of BC patients who underwent curative surgery. Next, we performed a prognostic analysis using BC patients who underwent curative surgery (blood: 330 cases, tissue: 307 cases of TCGA). First, we divided our cases into 2 groups using the minimum *p*-value approach (26) based on *PD-L1* mRNA expression, as described in the Materials and Methods section. The cut-off values of *PD-L1* mRNA expression in the blood and tumor tissues were found to be 2.74 and 5.61, respectively. The observation periods ranged from 0.06 to 11.6 years (median: 3.9 years) and from 0.03 to 11.0 years (median: 2.0 years), respectively. As shown in Figure 2A, BC patients with low *PD-L1* mRNA expression in either blood or tumor tissues had significantly poorer RFS than those with high expression ($p<0.05$; Figure 2A).

Next, univariate and multivariate regression analyses of predictive factors for RFS were performed (Table I). Univariate analyses showed that lymphatic involvement, lymph node metastasis, PgR-negativity and low *PD-L1* mRNA expression in the blood were statistically significant prognostic factors of RFS ($p=0.001$, $p=0.003$, $p=0.032$, and $p=0.019$, respectively). *PD-L1* mRNA expression in the blood and clinicopathological factors such as pathological tumor size, lymph node metastasis, PgR status, and HER2 status were included in the multivariate analysis. Notably, according to the multivariate analysis, low *PD-L1* mRNA expression in the blood was an independent prognostic factor of RFS in patients with BC (HR=4.73, $p=0.006$).

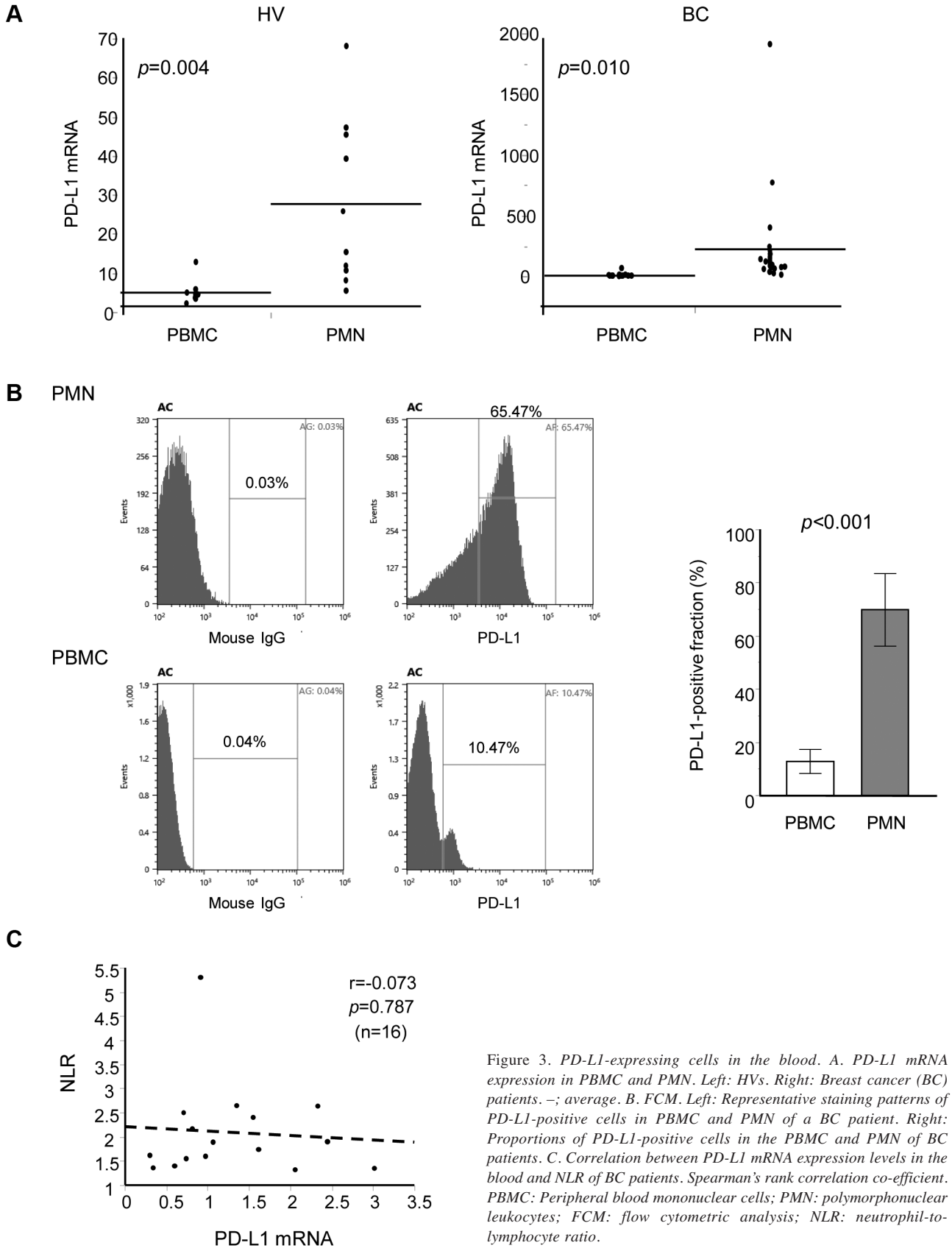

Figure 3. *PD-L1-expressing cells in the blood. A. PD-L1 mRNA expression in PBMC and PMN. Left: HVs. Right: Breast cancer (BC) patients. –; average. B. FCM. Left: Representative staining patterns of PD-L1-positive cells in PBMC and PMN of a BC patient. Right: Proportions of PD-L1-positive cells in the PBMC and PMN of BC patients. C. Correlation between PD-L1 mRNA expression levels in the blood and NLR of BC patients. Spearman's rank correlation co-efficient. PBMC: Peripheral blood mononuclear cells; PMN: polymorphonuclear leukocytes; FCM: flow cytometric analysis; NLR: neutrophil-to-lymphocyte ratio.*

Moreover, subgroup analyses for RFS were performed according to each intrinsic subtype. As shown in Figure 2B, patients with a low *PD-L1* mRNA expression in the blood exhibited poorer prognosis if they belonged in the HER2-positive group (p=0.037) compared to other groups (HR+/HER2– or TN).

Finally, in the subgroup analyses defined by TNM stages, there were no differences in RFS between high and low expression groups of *PD-L1* mRNA expression in the blood (Figure 3C).

Clinicopathological significance of PD-L1 mRNA expression in the blood. The relationship between clinicopathological factors and *PD-L1* mRNA expression in the blood was examined using the 330 patients with IDC who underwent curative surgery. As shown in Table II, no significant difference was found between the high and low *PD-L1* mRNA expression groups regarding clinicopathological factors such as age, pathological tumor size, nuclear grade, venous involvement, lymphatic involvement, lymph node metastasis or the status of ER, PgR and HER2.

PD-L1-expressing cells in the blood. *PD-L1* is expressed in tumor cells or immune cells including T cells, B cells, macrophages, dendritic cells and neutrophils (27-30). The PMN class of cells includes neutrophils, the most abundant of the PMN, and eosinophils, basophils, and mast cells. In contrast, the PBMC include monocytes and lymphocytes (T cells, B cells, and NK cells). Tumor cells are found in both fractions. First, we assessed the distribution of *PD-L1* expression in the blood of HVs or BC patients by RT-qPCR (Figure 3A). *PD-L1* mRNA expression in PMN was significantly higher compared with PBMC from either HVs or BC patients (p<0.05). In order to determine which blood cell subset expressed *PD-L1*, we performed FCM with a PD-L1 antibody using PMN or PBMC of 6 BC patients (Figure 3B). Representative data are shown in Figure 3B (Left). As expected, the number of PD-L1-positive cells was higher in PMN than PBMC [p<0.001, Figure 3B (Right)]. The results obtained from RT-qPCR and FCM indicate that PD-L1 is mainly expressed by PMN in the blood.

Correlation between PD-L1 mRNA expression levels in the blood and NLR of BC patients who underwent curative surgery. We examined the correlation between *PD-L1* mRNA expression levels in the blood and NLR, which is one of the indicators of the general immune status (31) in 16 BC patients. Spearman's rank correlation co-efficient demonstrated no correlation between them (r=0.073, p=0.787) (Figure 3C).

Table II. *Relationship between clinicopathological factors and PD-L1 mRNA expression levels in the blood of breast cancer patients*.

Variables	Low (n=194) Number (%)	High (n=136) Number (%)	p-Value
Age (y.o)			0.4162
<65	144 (74.2)	103 (75.7)	
≥65	46 (23.7)	26 (19.1)	
unknown	4 (2.1)	7 (5.2)	
Pathological tumor size			0.0936
1	94 (48.5)	79 (58.1)	
2, 3	100 (51.5)	57 (41.9)	
Nuclear grade			0.6201
1, 2	131 (67.5)	83 (61.0)	
3	57 (29.4)	41 (30.1)	
Unknown	6 (3.1)	12 (8.8)	
Venous involvement			0.3379
(–)	181 (93.3)	121 (89.0)	
(+)	9 (4.6)	10 (7.4)	
Unknown	4 (2.1)	5 (3.7)	
Lymphatic involvement			0.6400
(–)	117 (60.3)	86 (63.2)	
(+)	73 (37.6)	47 (34.6)	
Unknown	4 (2.1)	3 (2.2)	
Lymph node metastasis			1.0000
(–)	123 (63.4)	86 (63.2)	
(+)	71 (36.6)	49 (36.0)	
Unknown	0 (0.0)	1 (0.7)	
ER			0.7003
(–)	51 (26.3)	33 (24.3)	
(+)	140 (72.2)	103 (75.7)	
Unknown	3 (1.5)	0 (0)	
PgR			0.8202
(–)	81 (41.8)	55 (40.4)	
(+)	111 (57.2)	81 (59.6)	
Unknown	2 (1.0)	0 (0)	
HER2			0.3268
–(0, 1)	112 (57.7)	92 (67.6)	
+(2, 3)	61 (31.4)	39 (28.7)	
Unknown	21 (10.8)	5 (3.7)	

Correlation was analyzed by Fisher's exact test.

Discussion

Here, we showed that *PD-L1* mRNA expression in the blood could function as a biomarker that predicts recurrence of BC. Moreover, *PD-L1* mRNA expression in the blood is well correlated with that in tumor tissues of BC patients. To our knowledge, this is the first study to focus on the clinical significance of *PD-L1* mRNA expression in the blood as a liquid biopsy and to show a correlation between *PD-L1* mRNA expression in the blood and tumor tissues.

PD-L1 is expressed in tumor cells or immune cells, including T cells, B cells, macrophages, dendritic cells and neutrophils (27-30). Notably, we observed that *PD-L1* was mainly expressed in PMN in the blood and that low expression of *PD-L1* mRNA in the blood was an independent factor predicting recurrence in patients with primary BC, especially those with the HER2-positive type, as previously reported (32). This is interesting because *PD-L1* mRNA

expression in the blood was not associated with any clinicopathological factors. These findings suggested the hypothesis that *PD-L1* expression in the blood reflects host immune activation, which can suppress systemic metastasis of disseminated tumor cells (33). Thus, we examined whether *PD-L1* mRNA expression was associated with NLR, which is an indicator of the patients' general immune status (31). However, there was no association between them. Further studies are required to clarify the biological significance of *PD-L1* mRNA expression in the blood.

There is growing evidence that high *PD-L1* expression in tumor tissues is positively correlated with a more favorable prognosis, although this remains an imperfect predictor of ICI response (14, 32, 34, 35). In clinical treatment of breast cancer, combination therapy using atezolizumab (anti-PD-L1 antibody) and nab-paclitaxel has been used for patients with PD-L1-positive advanced TNBC, based on the results of IMpassion130 (NCT02425891) (10, 11). That is, the amount of PD-L1 expression in tumor tissues is a predictive biomarker for treatment with atezolizumab. Of note, we found that *PD-L1* mRNA expression in the blood is well correlated with that in tumor tissues of BC patients. These findings provide evidence that *PD-L1* mRNA expression in the blood can be used as a biomarker to predict the effect of atezolizumab instead of its expression in TNBC tumor tissues. Furthermore, *PD-L1* mRNA expression in the blood may have advantages in clinical use because it provides real-time information, is less invasive and cost effective compared to tumor tissues biopsies.

Unexpectedly, there was no statistically significant difference in the *PD-L1* mRNA expression levels between HVs and BC patients, intrinsic subtypes and TNM stages in the blood as well as in tumor tissues, although PD-L1 protein is reported to be highly expressed in high grade and aggressive intrinsic subtypes (TNBC or HER2-positive BC) (36-38). *PD-L1* mRNA expression might not be affected by tumor characteristics in BC.

In this study, we could not analyze the correlation between *PD-L1* mRNA expression in the blood of 330 BC patients and their paired tumor tissues because tumor tissues from the same patient cohort were not available. We are now conducting a study to test whether *PD-1* or *PD-L1* mRNA expression in TNBC patients' blood could be a biomarker for predicting the therapeutic effect of atezolizumab.

In summary, we demonstrated that low *PD-L1* mRNA expression in preoperative blood could be a marker of poor prognosis for BC patients. Furthermore, *PD-L1* mRNA expression in the blood was well correlated with tumor tissues of BC patients who underwent curative surgery. *PD-L1* mRNA expression in the blood may provide a convenient alternative biomarker to immunohistochemical staining of PD-L1 for predicting the therapeutic efficacy of PD-L1-targeted treatment. A large-scale prospective study is needed in order to study this further.

Conflicts of Interest

None of the Authors has a conflict of interest to disclose regarding this study.

Authors' Contributions

TM wrote the manuscript. MN and TM conducted the process of experiments. TM, MN, AT, GH, AF, SI, KK, YA, YM, HO, HU, SO, and KM designed the study and data interpretation. MN and TM prepared the organization of figures and statistical analysis.

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