

# Soluble PD-L1 Expression in Circulation as a Predictive Marker for Recurrence and Prognosis in Gastric Cancer: Direct Comparison of the Clinical Burden Between Tissue and Serum PD-L1 Expression

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## ABSTRACT

**Background.** This study assessed programmed cell death ligand 1 (PD-L1) expression in primary tissues and soluble PD-L1 (sPD-L1) concentration in matched preoperative serum in gastric cancer (GC) patients to perform direct comparison between tissue and serum PD-L1 expression and to clarify the prognostic implication in GC.

**Methods.** The study enrolled 180 GC patients who underwent surgery for GC at the authors' institution. The study evaluated tissue PD-L1 expression using immunohistochemistry and quantified sPD-L1 concentration in preoperative serum using enzyme-linked immunosorbent assay in GC patients.

**Results.** The findings showed that PD-L1 was overexpressed in GC tissues compared with normal mucosa. Tissue PD-L1 expression was significantly higher in the GC patients with advanced T stage, presence of lympho-vascular invasion, lymph node metastasis, and peritoneal metastasis.

Furthermore, elevated tissue PD-L1 expression was significantly associated with poor prognosis for overall survival (OS) and disease-free survival (DFS). Serum sPD-L1 was significantly higher in the GC patients than in the healthy volunteers. Although serum sPD-L1 was not correlated with any clinicopathologic factors, the patients with high serum sPD-L1 showed poorer OS and DFS than those with low sPD-L1. Multivariate analyses showed that both elevated tissue PD-L1 and serum sPD-L1 were independent prognostic factors for poor OS [tissue PD-L1: hazard ratio (HR), 4.28; 95% confidence interval (CI), 1.43–12.8;  $P = 0.0094$  vs. serum sPD-L1: HR, 11.2; 95% CI, 3.44–36.7;  $P = 0.0001$ ] and poor DFS (tissue PD-L1: HR, 6.96; 95% CI, 2.48–19.6;  $P = 0.0002$  vs. serum sPD-L1: HR, 8.7; 95% CI, 3.16–23.9;  $P < 0.0001$ ) for the GC patients. Furthermore, infiltrative CD8- and Foxp3-positive T cells were significantly increased in the GC patients with elevated tissue PD-L1 expression.

**Conclusion.** Both serum sPD-L1 and tissue PD-L1 expression may serve as predictive biomarkers for recurrence and prognosis in GC patients.

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Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide.<sup>1–3</sup> To improve disease outcome, an urgent need exists for the development of prognostic and predictive biomarkers to identify appropriate GC patient groups for the most effective treatment choices.

The immune system can recognize cancer cells and suppress tumor development and the metastasis process.<sup>4–7</sup> Some studies have shown that tumor cells have the

capacity to escape immune detection and attack by the host immune responses through the use of diverse mechanisms.<sup>8,9</sup> Thus, identification of the mechanisms involved in the escape of immune suppression might help identify novel therapeutic targets for the development of more robust and targeted therapeutic regimens.<sup>10</sup> Indeed, several studies have established the success of immune checkpoint inhibitors in various cancers, including non-small cell lung carcinoma, renal cell carcinoma, melanoma, and GC.<sup>11–13</sup>

The programmed cell death ligand 1 (PD-L1)/programmed cell death receptor 1 (PD-1) pathway has been considered a promising target for cancer treatment based on its role in tumor immunity. Typically, PD-1 is expressed by activated lymphocytes and interacts with the PD-L1 ligand. Binding of PD-1 to a ligand results in the inhibition of the proliferation and activation of T cells, eventually leading to the immune evasion of tumor cells.<sup>14–17</sup>

Several studies have shown that PD-L1 overexpression is an indicator of a poor prognosis in several malignancies.<sup>18–23</sup> It is known that PD-L1 localizes to the cell surface of tumor and immune cells, and a recent study showed the existence of soluble PD-L1 (sPD-L1) released from PD-L1-positive cells in human serum.<sup>24</sup> Furthermore, several lines of evidence demonstrate the clinical burden of sPD-L1 in hematopoietic malignancies.<sup>25,26</sup>

Although several studies have shown the clinical burden and prognostic impact of tissue PD-L1 expression in GC patients, to the best of our knowledge, the clinical significance of circulating sPD-L1 in GC and direct comparison of the clinical burden between tissue and serum sPD-L1 have not been examined.

## MATERIALS AND METHODS

### *Patients and Sample Collection*

The study enrolled 180 GC patients (116 men and 64 women) who underwent surgery for GC at Mie University Hospital, Japan from 2008 to 2014. The patients were included according to the availability of matched tissue and serum samples with complete clinical data. Additional information is described in Supplementary Materials and Methods.

### *Immunohistochemical Analysis and Evaluation of PD-L1 Expression Scores*

Formalin-fixed, paraffin-embedded sections (5  $\mu$ m thick) were prepared from surgical specimens of the GC patients and incubated with an antibody against PD-L1 (diluted 1:100; 27A2, LS-C179481; LifeSpan Biosciences, Inc., Seattle, WA, USA) at 4 °C overnight.

Additional information is described in Supplementary Materials and Methods.

Each slide was observed by scanning of the entire tissue specimen at magnifications of  $\times 40$  and  $\times 200$ . Two pathologists without prior knowledge of patient clinicopathologic characteristics evaluated PD-L1 immunoreactivity at the core of GC according to the intensity and extent of staining. The evaluators used a multihead microscope to resolve discrepancies. Additional information is described in Supplementary Materials and Methods (Fig. S1).

### *Immunohistochemical Analysis to Detect Foxp3 and CD8 Expression in GC Tissues*

Formalin-fixed, paraffin-embedded (FFPE) specimens were sliced into 5- $\mu$ m sections and subjected to immunohistochemical analysis to detect Foxp3 and CD8 expression. The primary antibodies used were monoclonal mouse anti-human Foxp3 antibody (clone: 236A/E, dilution 1:100; Abcam, Cambridge, UK) for regulatory T cells and monoclonal rabbit anti-human CD8 (clone: EP1150, dilution 1:1000; GeneTex, San Antonio, TX, USA) for cytotoxic T cells. Additional information is described in Supplementary Materials and Methods.

### *Scoring Foxp3- and CD8-Positive T Cells*

Foxp3- and CD8-positive T cells were counted using a scanner system under an Olympus BX-51 and DP21 (Olympus, Tokyo, Japan) with Cellsens software imaging system, as previously described.<sup>27</sup> Additional information is described in Supplementary Materials and Methods.

### *Enzyme-Linked Immunosorbent Assay*

Serum sPD-L1 concentrations were determined using enzyme-linked immunosorbent assay kits for human PD-L1 (WLS Cloud-Clone Corp., Houston, TX, USA), as previously described.<sup>25,28</sup> Further information is described in Supplementary Materials and Methods.

### *Statistical Analysis*

Statistical analyses were performed using JMP version 10 (SAS Institute, Cary, NC, USA). The results are expressed as mean  $\pm$  standard deviation (SD). Receiver operating characteristic (ROC) curves were generated to determine cut-off values of tissue and serum PD-L1 expression for analysis of survival by Youden's Index (tissue PD-L1: score 6; serum sPD-L1: 0.507 ng/mL). Additional information is described in Supplementary

Materials and Methods. All  $P$  values were two-sided, and  $P$  values lower than 0.05 were considered statistically significant.

## RESULTS

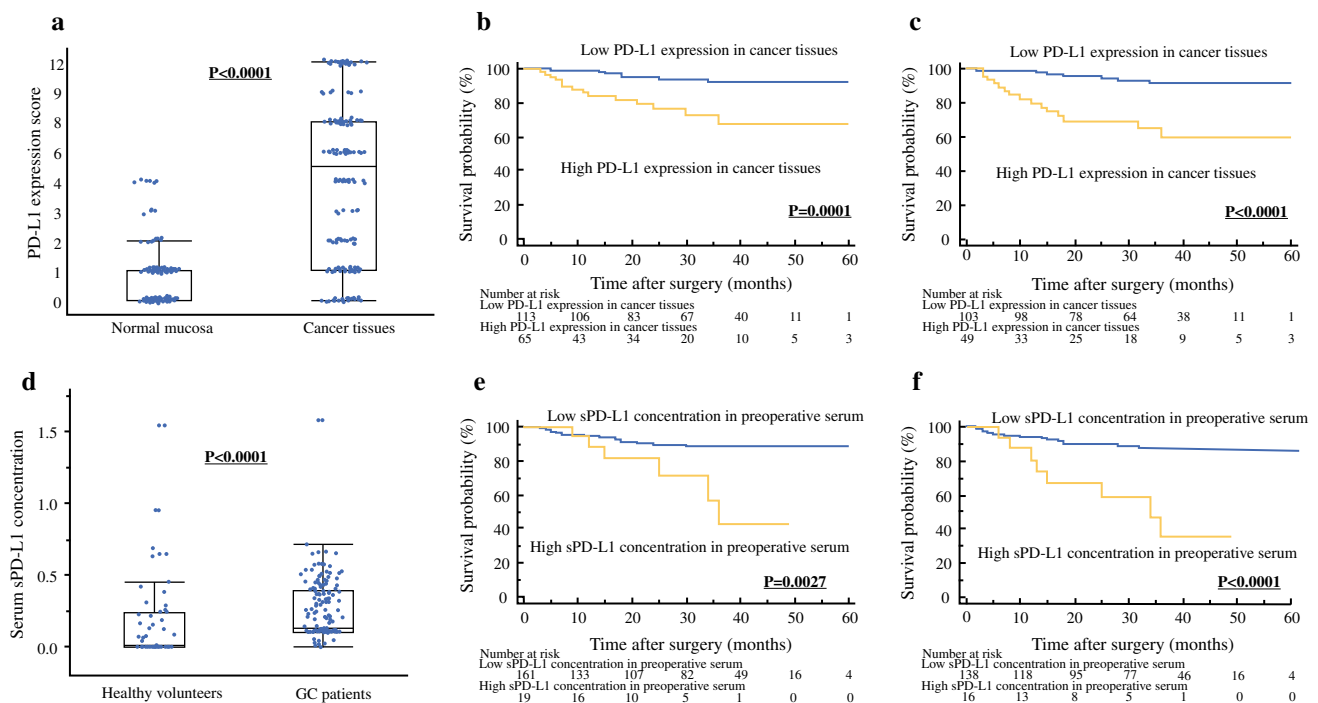
### Expression Pattern of PD-L1 in GC Tissues

To assess the expression pattern of PD-L1 in GC tissues, we performed immunohistochemical analysis of PD-L1 using GC tissues and adjacent normal mucosa and quantifying staining as described in the Methods section. We stained PD-L1 mainly in the cellular membrane of GC cells, consistent with previous reports for other types of cancers.<sup>29</sup> Notably, expression of PD-L1 was significantly upregulated in GC cells compared with adjacent normal mucosa ( $P < 0.0001$ , Wilcoxon rank correlation test; Fig. 1a, Fig. S1).

### High PD-L1 Expression Was Significantly Correlated With Clinicopathologic Factors in GC Patients

We next assessed the relationship between tissue PD-L1 expression and various clinicopathologic variables in the GC patients. Interestingly, higher PD-L1 expression in GC tissues was significantly correlated with well-established disease progression factors, including advanced T stage ( $P = 0.0003$ ), presence of vessel ( $P < 0.0001$ ) and lymphatic vessel involvement ( $P = 0.0005$ ), lymph node metastasis positivity ( $P = 0.023$ ), and peritoneal metastasis positivity ( $P = 0.0098$ ) in the GC patients (Table 1).

We next generated a Kaplan–Meier survival curve based on PD-L1 expression to perform the time-to-event analysis and evaluate the potential use of tissue PD-L1 expression as a prognostic biomarker. Interestingly, the patients with elevated PD-L1 expression in GC tissues had a significantly poorer prognosis than those with tissue PD-L1



**FIG. 1** **a–c** Programmed cell death ligand 1 (PD-L1) is highly expressed in gastric cancer (GC) tissues, and elevated PD-L1 expression in GC tissues was significantly correlated with poor overall survival (OS) and disease-free survival (DFS) for the GC patients. **a** PD-L1 expression was significantly increased in cancerous tissues compared with matched adjacent normal mucosa in 180 GC patients ( $P < 0.0001$ ). **b, c** Kaplan–Meier survival curves for OS and DFS for the GC patients based on PD-L1 expression in GC tissues. **b** The overall survival (OS) for the GC patients with high PD-L1 expression in GC tissues was significantly lower than for the patients with low PD-L1 expression ( $P = 0.0001$ , log-rank test). **c** The DFS for the GC patients with high PD-L1 expression in GC tissues was significantly lower than for the patients with low PD-L1 expression

( $P < 0.0001$ , log-rank test). **d–f** Serum soluble PD-L1 (sPD-L1) was significantly increased in the GC patients, and a high level of serum sPD-L1 was significantly correlated with poor OS and DFS for the GC patients. **d** The serum sPD-L1 concentration was significantly increased in the GC patients compared with the healthy control subjects ( $P < 0.0001$ ). **e, f** Kaplan–Meier survival curves for OS and DFS for the GC patients based on sPD-L1 levels in the preoperative serum of the GC patients. **e** The OS for the GC patients with high sPD-L1 levels was significantly lower than for those with low sPD-L1 levels in preoperative serum ( $P = 0.0027$ , log-rank test). **f** The DFS for the GC patients with high sPD-L1 levels was significantly lower than for those with low sPD-L1 levels in preoperative serum ( $P < 0.0001$ , log-rank test). All statistical tests were two-sided

**TABLE 1** Correlation between clinicopathologic variables and tissue programmed cell death ligand 1 (PD-L1) expression in gastric cancer (GC) patients

Variable	<i>n</i>	PD-L1 expression Mean $\pm$ SD	<i>P</i> value
Gender			
Male	116	5.65 $\pm$ 4.32	0.12
Female	64	4.75 $\pm$ 4.03	
Age (years) <sup>a</sup>			
$\geq 70$	100	4.79 $\pm$ 4.12	0.13
> 70	80	6.01 $\pm$ 4.29	
Histologic type			
Intestinal type	78	6.21 $\pm$ 4.24	0.014 <sup>b</sup>
Diffuse type	102	4.63 $\pm$ 4.14	
Pathologic T category			
pT1/2	108	4.33 $\pm$ 3.94	0.0003 <sup>b</sup>
pT3/4	72	6.83 $\pm$ 4.29	
Vessel invasion			
Present	63	7.17 $\pm$ 4.22	< 0.0001 <sup>b</sup>
Absent	117	4.34 $\pm$ 3.92	
Lymphatic vessel invasion			
Present	109	6.38 $\pm$ 4.31	0.0005 <sup>b</sup>
Absent	71	3.73 $\pm$ 3.60	
Lymph node metastasis			
Present	73	6.37 $\pm$ 4.37	0.023 <sup>b</sup>
Absent	107	4.63 $\pm$ 4.01	
Peritoneal metastasis			
Present	13	7.84 $\pm$ 4.02	0.0098 <sup>b</sup>
Absent	167	5.14 $\pm$ 4.20	
Distant metastasis			
Present	15	6.60 $\pm$ 4.61	0.42
Absent	165	5.22 $\pm$ 4.19	
UICC TNM classification			
Stage 1	98	3.87 $\pm$ 3.60	
Stage 2	27	5.38 $\pm$ 4.20	
Stage 3	29	8.24 $\pm$ 4.00	
Stage 4	26	7.11 $\pm$ 4.37	

SD standard deviation, UICC Union for International Cancer Control, TNM tumor-node-metastasis

<sup>a</sup>The median age at surgery is 70 years for GC patients

<sup>b</sup>*P* < 0.05

expression under the cut-off point in terms of OS in the GC cohort (*P* = 0.0001, log-rank test; Fig. 1b). Furthermore, increased PD-L1 expression in GC tissues was significantly correlated with poorer disease-free survival (DFS) than low PD-L1 expression (*P* < 0.0001, log-rank test; Fig. 1c).

### Concentration of sPD-L1 in Preoperative Serum From GC Patients

Based on the finding of PD-L1 overexpression in GC tissues, we examined the sPD-L1 levels in tissue-matched preoperative serum specimens from the GC patients and the healthy volunteers to clarify the diagnostic potential of sPD-L1 in GC. The sPD-L1 concentration in preoperative serum was significantly higher in the GC patients than in the healthy control subjects (*P* < 0.0001; Fig. 1d). None of the clinicopathologic factors except lymphatic vessel invasion were correlated with the preoperative serum sPD-L1 levels in the GC patients (Table 2). However, the GC patients with high sPD-L1 levels in preoperative serum showed poorer OS and DFS than the patients with low serum sPD-L1 levels (*P* = 0.0027 and *P* < 0.0001, respectively; Fig. 1e and f).

### High sPD-L1 Level in Preoperative Serum was an Independent Prognostic Factor for Both OS and DFS in GC Patients

We next performed a multivariate Cox regression analysis to elucidate the potential of prognostic biomarkers in the GC patients. The univariate analysis showed that the risk factors for poor OS were advanced T stage (*P* = 0.0006), vessel involvement (*P* = 0.0012), lymphatic vessel involvement (*P* = 0.016), lymph node metastasis (*P* = 0.0002), peritoneal metastasis (*P* < 0.0001), distant metastasis (*P* < 0.0001), high tissue PD-L1 expression (*P* = 0.0006), and high serum PD-L1 concentration (*P* = 0.0054) (Table 3a).

In addition, the multivariate analysis showed that the risk factors for poor OS were lymph node metastasis [hazard ratio (HR), 9.67; 95% confidence interval (CI), 1.95–47.9; *P* = 0.0054], peritoneal metastasis (HR, 3.84; 95% CI, 1.17–12.5; *P* = 0.026), distant metastasis (HR, 24.7; 95% CI, 7.6–79.9; *P* < 0.0001), high tissue PD-L1 expression (HR, 4.28; 95% CI, 1.43–12.8; *P* = 0.0094), and high serum PD-L1 concentration (HR, 11.2; 95% CI, 3.44–36.7; *P* = 0.0001) (Table 3a). Notably, the multivariate analysis for DFS showed that the independent risk factors for poor DFS were high tissue PD-L1 expression (HR, 6.96; 95% CI, 2.48–19.6; *P* = 0.0002) and high serum PD-L1 concentration (HR, 8.7; 95% CI, 3.16–23.9; *P* < 0.0001) (Table 3b).

Collectively, these data suggest that both tissue PD-L1 expression and sPD-L1 levels in preoperative serum could be used as predictive biomarkers for poor oncologic outcome and for the identification of a GC population at high risk for recurrence.

**TABLE 2** Correlation between clinicopathologic variables and serum soluble programmed cell death ligand 1 (sPD-L1) levels in gastric cancer (GC) patients

Variable	<i>n</i>	sPD-L1 levels Mean $\pm$ SD	<i>P</i> value
Gender			
Male	116	0.26 $\pm$ 0.21	0.76
Female	64	0.24 $\pm$ 0.17	
Age (years) <sup>a</sup>			
$\geq 70$	100	0.24 $\pm$ 0.20	0.55
$> 70$	80	0.26 $\pm$ 0.19	
Histologic type			
Intestinal type	78	0.26 $\pm$ 0.23	0.95
Diffuse type	102	0.245 $\pm$ 0.17	
Pathologic T category			
pT1/2	108	0.25 $\pm$ 0.21	0.54
pT3/4	72	0.24 $\pm$ 0.18	
Vessel invasion			
Present	63	0.23 $\pm$ 0.17	0.44
Absent	117	0.26 $\pm$ 0.21	
Lymphatic vessel invasion			
Present	109	0.23 $\pm$ 0.17	0.023 <sup>b</sup>
Absent	71	0.28 $\pm$ 0.23	
Lymph node metastasis			
N0	107	0.27 $\pm$ 0.21	0.096
N1	73	0.22 $\pm$ 0.17	
Peritoneal metastasis			
P0	167	0.25 $\pm$ 0.01	0.77
P1	13	0.25 $\pm$ 0.19	
Distant metastasis			
M0	165	0.25 $\pm$ 0.20	0.18
M1	15	0.16 $\pm$ 0.13	
UICC TNM classification			
Stage 1	98	0.26 $\pm$ 0.21	0.18
Stage 2	27	0.25 $\pm$ 0.16	
Stage 3	29	0.22 $\pm$ 0.18	
Stage 4	26	0.21 $\pm$ 0.17	

SD Standard deviation, UICC union for international cancer control, TNM tumor-node-metastasis

<sup>a</sup>The median age at surgery is 70 years for GC patients

<sup>b</sup> $P < 0.05$

### Correlation Between Tissue PD-L1 and Serum sPD-L1 Levels in GC Patients

We assessed the correlation between tissue PD-L1 and serum sPD-L1 levels in the GC patients. Although preoperative serum sPD-L1 showed a tendency toward elevation in the GC patients with high tissue PD-L1 expression compared with those who had low tissue PD-L1

expression, a significant correlation was not observed ( $P = 0.1$ ; Fig. S2).

### Immunohistochemical Analysis of Tumor-Infiltrating Foxp3- and CD8-Positive T Cells

Finally, we evaluated the magnitude of tumor-infiltrating lymphocytes (TILs) by counting the numbers of intratumoral Foxp3- and CD8-positive T cells to clarify the correlation between TILs and tissue or serum PD-L1 expression in the GC patients (Fig. S3). In the marginal part, the median numbers of CD8- and Foxp3-positive T cells were respectively 57 (range, 3.33–165.6) and 42.6 (range, 5.33–135.3). Although we did not find any significant correlation between preoperative serum sPD-L1 levels and TILs, infiltrative CD8- and Foxp3-positive T cells were significantly increased in the GC patients with elevated tissue PD-L1 expression ( $P = 0.037$  and  $P = 0.0006$ , respectively; Table 4).

## DISCUSSION

This study investigated tissue PD-L1 expression and serum sPD-L1 levels using matched specimens from GC patients and made several novel discoveries. First, we assessed PD-L1 expression in GC tissues from 180 GC patients and showed that PD-L1 was significantly overexpressed in GC tissues compared with adjacent normal mucosa. Overexpression of PD-L1 was significantly correlated with local disease progression factors, including advanced depth of invasion, lymphatic vessel, and vascular invasion in GC patients. Second, the patients with high PD-L1 expression in GC tissues showed poorer prognosis of both OS and DFS than those with low PD-L1 expression. Third, the serum sPD-L1 levels in the GC patients were significantly higher than in the healthy volunteers, and the high levels of serum sPD-L1 in the GC patients were significantly correlated with poorer OS and DFS than for the patients with low serum sPD-L1 levels. Finally, the multivariate analysis clearly showed that both the high levels serum sPD-L1 and the high PD-L1 expression in GC tissues were independent prognostic factors for both OS and DFS in the GC patients.

As a type 1 transmembrane protein and as a member of the CD28/CTLA-4 immunoglobulin family,<sup>30</sup> PD-1 is expressed on various types of immune cells, including monocytes, dendritic cells, natural killer cells, B cells, T cells, and many tumor-infiltrating lymphocytes (TILs).<sup>17</sup> Also, PD-1 is one of the most important inhibitory co-receptors expressed by T cells via activation of an immunoreceptor tyrosine-based switch motif.<sup>31</sup> Furthermore, PD-1 also is expressed on regulatory T cells and can



**TABLE 3** Prognostic impact of serum sPD-L1 level in gastric cancer (GC) patients. Multivariate analysis for (a) predictors of overall survival, (b) predictors of disease-free survival

Variables	Univariate			Multivariate		
	HR	95% CI	P value	HR	95% CI	P value
<i>(a)</i>						
Gender (male)	1.75	0.64–4.78	0.27			
Age (> 70 years) <sup>a</sup>	1.68	0.71–3.97	0.24			
Histologic type (diffuse type)	1.98	0.77–5.11	0.16			
T classification (pT3/4)	5.85	2.14–16	0.0006 <sup>b</sup>	0.91	0.19–4.47	0.91
Vessel involvement (present)	4.48	1.8–11.1	0.0012 <sup>b</sup>	0.97	0.29–3.18	0.96
Lymphatic vessel involvement (present)	4.48	1.32–15.2	0.016 <sup>b</sup>	0.64	0.11–3.81	0.62
Lymph node metastasis (present)	10.4	3.06–35.3	0.0002 <sup>b</sup>	9.67	1.95–47.9	0.0054 <sup>b</sup>
Peritoneal metastasis (present)	13.9	5.47–35.5	<0.0001 <sup>b</sup>	3.84	1.17–12.5	0.026 <sup>b</sup>
Distant metastasis (present)	19.8	8.02–49.1	<0.0001 <sup>b</sup>	24.7	7.6–79.9	< 0.0001 <sup>b</sup>
Tissue PD-L1 expression (score > 6)	4.98	2.0–12.4	0.0006 <sup>b</sup>	4.28	1.43–12.8	0.0094 <sup>b</sup>
Serum sPD-L1 level (> 0.507 ng/mL)	3.87	1.49–10	0.0054 <sup>b</sup>	11.2	3.44–36.7	0.0001 <sup>b</sup>
<i>(b)</i>						
Gender (male)	2.01	0.74–5.44	0.17			
Age (> 70 years) <sup>a</sup>	1.9	0.82–4.41	0.13			
Histologic type (diffuse type)	2.87	1.06–7.77	0.039 <sup>b</sup>	1.76	0.56–5.52	0.33
T classification (pT3/4)	11.6	3.91–34.2	<0.0001 <sup>b</sup>	3.62	0.91–14.4	0.069
Vessel involvement (present)	4.09	1.75–9.58	0.0012 <sup>b</sup>	0.93	0.36–2.4	0.87
Lymphatic vessel involvement (present)	9.16	2.14–39.2	0.0028 <sup>b</sup>	1.36	0.19–9.83	0.76
Lymph node metastasis (present)	10.7	3.62–31.7	<0.0001 <sup>b</sup>	4.5	1.1–18.3	0.036 <sup>b</sup>
Tissue PD-L1 expression (score > 6)	6.39	2.6–15.7	0.0001 <sup>b</sup>	6.96	2.48–19.6	0.0002 <sup>b</sup>
Serum sPD-L1 level (> 0.507 ng/mL)	5.78	2.41–13.8	0.0001 <sup>b</sup>	8.7	3.16–23.9	< 0.0001 <sup>b</sup>

HR Hazard ratio, CI confidence interval, PD-L1 programmed cell death ligand 1, sPD-L1 soluble PD-L1

<sup>a</sup>The median age at surgery is 70 years for gastric cancer patients

<sup>b</sup>P < 0.05

**TABLE 4** Correlation between tumor infiltrative lymphocytes and tissue and serum programmed cell death ligand 1 (PD-L1) levels in gastric cancer (GC) patients

Variable	<i>N</i>	Tissue PD-L1 expression Mean ± SD	<i>P</i> value	Serum sPD-L1 levels Mean ± SD	<i>P</i> value
<i>CD8-positive TILs<sup>a</sup></i>					
High	90	6.08 ± 4.08	0.037b <sup>b</sup>	0.24 ± 0.21	0.55
Low	90	4.57 ± 4.27		0.25 ± 0.18	
<i>Foxp3-positive TILs<sup>a</sup></i>					
High	89	6.55 ± 3.89	0.0006b <sup>b</sup>	0.23 ± 0.18	0.1
Low	91	4.14 ± 3.89		0.27 ± 0.22	

sPD-L1 Soluble PD-L1, SD standard deviation, TIL tumor-infiltrating lymphocyte

<sup>a</sup>Cutoff points of CD8- and Foxp3-positive TILs were determined by the median value of each covariate

<sup>b</sup>P < 0.05

enhance the proliferation of regulatory T cells and restrain the immune response.<sup>32</sup> Both PD-L1 (also named B7-H1; CD274) and PD-L2 (B7-DC; CD273) have been identified as ligands of PD-1. Whereas PD-L2 is expressed on macrophages and dendritic cells, PD-L1 is broadly expressed on various types of cells, including B cells, T cells, dendritic cells, macrophages, and vascular endothelial cells, and in various malignancies.<sup>18–23,33,34</sup>

To date, several lines of evidence have demonstrated the prognostic burden of tissue PD-L1 expression in malignancies, including in GC.<sup>35–38</sup> Recently, Ju et al.<sup>39</sup> evaluated PD-L1 expression and TILs in primary tissues from 105 GC patients and demonstrated that PD-L1 expression in tumor cells was significantly associated with poor prognosis in GC patients. Furthermore, overexpression of PD-L1 with low-density CD3+ and CD8+ TILs

showed a shorter OS than high-density CD3+ and/or CD8+ TILs, and these results suggested that PD-L1 might be deeply involved in disease development via modulation of host tumor immunity in primary tumor sites. Consistent with these results, our study clearly showed that high PD-L1 expression in GC tissues was significantly correlated with local disease-progression factors, high density of both CD8- and Foxp3-positive TILs, and poor prognosis of OS and DFS in GC patients.

Another major finding of this study was the clinical burden of serum sPD-L1 demonstrated by direct comparison between tissue PD-L1 expression and serum sPD-L1 levels in GC patients. Based on recent studies showing the clinical burden of tissue PD-L1 expression in malignancies, circulating sPD-L1 was demonstrated to be a prognostic biomarker in several types of cancer.<sup>25,26</sup> Whereas PD-L1 is expressed on the membrane of tumor cells, circulating sPD-L1 is thought to be released from PD-L1-positive tumor cells or immune cells. Chen et al.<sup>24</sup> previously evaluated PD-L1 concentration in supernatants from various cell lines with or without PD-L1 expression and demonstrated that PD-L1 was detectable in supernatants from PD-L1(+) cells but not from PD-L1(-) cell lines. In line with this evidence, our study showed that circulating sPD-L1 concentration was significantly increased in GC patients compared with healthy volunteers.

Several studies have demonstrated the feasibility of circulating sPD-L1 as a prognostic marker in patients with malignancies. Ha et al.<sup>28</sup> evaluated sPD-L1 concentration in serum specimens from 158 patients with biliary tract cancer and showed that high sPD-L1 was one of the independent poor prognostic factors for advanced biliary tract cancer patients treated with palliative chemotherapy. More recently, Amatatsu et al.<sup>40</sup> quantified circulating PD-L1 mRNA expression using peripheral blood specimens from 124 GC patients and showed that elevated expression of PD-L1 mRNA in peripheral blood is an independent prognostic factor in GC patients.

In our study, despite the lack of statistical correlation between preoperative serum sPD-L1 levels and clinicopathologic factors, the GC patients with high sPD-L1 levels in preoperative serum showed poorer OS and DFS than those with low serum sPD-L1 levels. Interestingly, the multivariate analysis showed that both high serum sPD-L1 concentration and tissue PD-L1 expression were independent risk factors for both poor OS and poor DFS.

We should consider two major points from this study. The first major point is the source of the circulating sPD-L1 in the GC patients. Although our study showed an increase of PD-L1 expression in both cancer tissues and GC patients, circulating sPD-L1 concentration was not significantly correlated with tissue PD-L1 expression in the GC patients. Previous evidence suggested that circulating sPD-

L1 in patients with malignancies might arise from multiple sources produced by distinct mechanisms such as intrinsic splicing activities in tumor cells, pro-tumor inflammatory responses, and antitumor immune responses. These multiple sources might play an influential role in the circulating sPD-L1 concentration in GC patients.

The second major point is the functional role of circulating sPD-L1 in GC patients. Accumulating evidences have shown that PD-L1/PD-1 binding in the tumor microenvironment suppresses the activation of T cells and immune evasion through recruitment of SHP-2.<sup>41</sup> Furthermore, findings also show that soluble PD-L1 is involved in tumor-associated immune suppression and host-immune damage via inhibition of T cell activation.<sup>25,34</sup>

Considering these evidences, our findings suggested that PD-L1 expression in cancer tissues could reflect the local disease development in the tumor site, and sPD-L1 might reflect the systemic immunologic status for malignancies in the host. Therefore, serum PD-L1 concentration was not significantly correlated with clinicopathologic variables about tumor stage. On the other hand, PD-L1 expression status in primary tissues was significantly correlated with clinicopathologic variables reflect for local development, including advanced tumor depth and presence of lymphovascular invasion.

In conclusion, this study showed that PD-L1 expression in cancer tissues and preoperative sPD-L1 concentration have different clinical significances in GC, and that both circulating sPD-L1 and tissue PD-L1 expression might be used as prognostic biomarkers in GC patients.

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