Expression patterns of programmed death-1 and programmed death-1 ligand-1 on T cells in gastric cancer

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Abstract. The aim of the present study was to evaluate programmed death-1 (PD-1) and programmed death-1 ligand-1 (PD-L1) expression in gastric carcinoma and to assess their effect on survival rate. A total of 170 surgically resected specimens were obtained from patients diagnosed with gastric carcinoma at St. Vincent's Hospital, The Catholic University of Korea. Paraffin tissue sections from tissue microarray blocks were subjected to immunohistochemical analysis of PD-1 and PD-L1. In addition, PD-1 expression on $CD4^+$ and $CD8^+$ T cells isolated from peripheral blood mononuclear cells and gastric cancer tissues was evaluated by multicolor flow cytometry. PD-1 and PD-L1 were expressed in 30.0 and 60.5% of the gastric cancer tissues, respectively. The expression of PD-L1 was higher in patients with advanced T (P=0.035) and Tumor, Node and Metastasis stage (P=0.05). The patients with positive PD-L1 expression had shorter disease-free survival time than those without PD-L1 expression (P=0.005). Additionally, PD-L1 expression was significantly associated with poor prognosis (P=0.015). PD-1 and PD-L1 expression levels were significantly higher on $CD8^+$ T cells than on $CD4^+$ T cells (P<0.001). The data of the present study suggested that PD-L1 expression may be an independent indicator of poor prognosis in patients with gastric cancer. Furthermore, PD-L1 expression may play a role in immune evasion of gastric cancer.

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Introduction

Gastric cancer is the fourth most frequent malignancy and the second most frequent cause of cancer-associated mortality worldwide (1). Although curative resection offers the best prognosis, a substantial number of patients experience recurrence or metastasis even after R0 resection due to micrometastasis (2). Adjuvant chemotherapy after curative gastrectomy reportedly increases survival and controlled micrometastasis (3). Immunotherapies, such as immune-checkpoint inhibitors, are emerging alternatives for controlling cancer micrometastasis and improving the prognosis of patients with malignancies (4-7).

Tumor-infiltrating immune cells are found in various malignancies; therefore, immunological markers may be predictive of the prognosis of patients with cancer (8-10). T cells are involved in the recruitment and activation of effector cells and amplification of the specific immune response to pathogens and cancer cells (11). Cancer cells express tumor antigens, which makes them susceptible to recognition and lysis by T cells (12). Programmed death-1 (PD-1) is an immunoinhibitory receptor expressed by chronically stimulated $CD4^+$ and $CD8^+$ T cells after activation (13,14). The interaction between PD-1 and its ligand, PD-1 ligand-1 (PD-L1), contributes to the maintenance of peripheral tolerance to self-antigens in normal hosts (15). PD-L1 is expressed by various solid tumors, such as renal cell carcinoma, breast cancer, pancreatic cancer, colorectal cancer, and esophageal cancer and is associated with a diminished antitumor T cell response (4,6,16) In addition, PD-L1 expression is associated with a poor prognosis in patients with various solid malignancies, such as esophageal cancer, pancreatic cancer, and gastric carcinoma (7,17,18). Despite the expression of tumor rejection antigens and tumor-specific cytotoxic T cells, the immune system fails to mount a response against gastric cancer; however, the mechanisms underlying this immune evasion are unclear (19).

In the present study, an immunohistochemical analysis of PD-1 and PD-L1 was conducted using gastric cancer tissue microarrays (TMAs) and PD-1 and PD-L1 expression in T cells in gastric cancer was evaluated. The association among expression levels of PD-1 and PD-L1, clinicopathological factors and survival were also analyzed.

Patients and methods

Patients. Between January 2004 and December 2012, 170 patients who were diagnosed with gastric adenocarcinoma and received gastrectomy at St. Vincent's Hospital, The Catholic University of Korea (Suwon, Republic of Korea), were retrospectively analyzed. Patients with a history of other malignancies or recurrent tumors were excluded. In total, 170 formalin-fixed paraffin-embedded gastric adenocarcinoma tissue samples isolated from patients who underwent surgical treatment at St. Vincent's Hospital, The Catholic University of Korea between January 2004 and December 2012 were analyzed in the present study. The study protocol was approved by the Institutional Review Board at St. Vincent's Hospital, The Catholic University of Korea (Suwon, Republic of Korea). Clinical data, including age, sex, overall survival (OS) time, metastasis and recurrence, were obtained through medical chart reviews. Pathological data, including Tumor-Node-Metastasis (TNM) stage (20), histologic type according to Lauren classification (20), and presence of perivascular invasion were evaluated by immunohistochemistry. The surgical treatment comprised of gastric resection, according to the localization of the primary tumor, and lymph node dissection, in line with the recommendations of the Japanese Research Society for Gastric Cancer (21). All tissues were examined by a pathologist and were classified in accordance with the guidelines of the Japanese Classification of Gastric Carcinoma (20).

For flow cytometry, an additional 30 gastric cancer tissue samples from another sample of patients were obtained from resected specimens between January and March 2017. This sample of patients included 19 men and 11 women, and the mean age was 64 years (range, 40-84 years). Peripheral blood (~30 ml) was collected from patients prior to surgery. The gastric tumor samples were freshly frozen in liquid nitrogen immediately after surgical resection. The blood and the tumor samples were kept at -70˚C until flow cytometry was performed. The study protocol was approved by the Institutional Review Board at St. Vincent's Hospital, The Catholic University of Korea (Suwon, Republic of Korea). Patients whose tissues were used for flow cytometry provided written informed consent. The follow-up program consisted of computed tomography scans, endoscopic examination, blood tests and chest radiography at 6-month intervals. OS was defined as the time between the date of diagnosis and the mortality date from any cause or the date of the last follow-up visit. Patients deceased due to surgical treatments or other causes were excluded from the present study. Disease-free survival (DFS) was defined as the time from tumor resection to the earlier of the following outcomes: i) Disease recurrence (loco-regional or metastatic); ii) last follow-up without evidence of disease; or iii) death without evidence of disease.

Construction of the tissue microarray block. All surgical specimens were fixed in 10% buffered formalin at 4˚C for 24-48 h and embedded in paraffin. Single representative core biopsy specimens with a diameter of 2-mm were taken from the tumor blocks using a core biopsy tool (SeongKohn Trader's Corp.), arranged on a new TMA mould, and re-embedded in paraffin. The TMA blocks, containing 60 cores, were cut into 4-μm-thick sections and stained at room temperature with 0.5% haematoxylin for 4 min and 1% eosin for 30 sec. The tissues were then examined under a high-power field light microscope (magnification, x400) and tumors occupying >10% of the core area were selected as tumor sites.

Immunohistochemistry. Sections (thickness, 4 μm) from the TMA blocks were mounted on Superfrost glass slides, deparaffinised in xylene, and rehydrated in a graded series of ethanol (100, 95, 90, 80, 70% ethanol). To block endogenous peroxidase activity, the tissue slides were incubated with 3% H_2O_2 for 15 min. Tissue slides were subsequently heated at 100˚C for 25 min in a microwave in EDTA (pH 8.0) for antigen retrieval. The sections were incubated overnight at 4˚C with 1:100 dilutions of primary antibodies against PD-1 (cat. no. ab52587; Abcam) and PD-L1 (cat. no. ab58810; Abcam). Immunostaining was conducted using the ImmPRESS (cat. no. MP7401-50; Vector Laboratories, Inc.) system and the 3,3'-diaminobenzidine kit (cat. no. Sk4100; Vector Laboratories, Inc.). The sections were counterstained with 0.5% Meyer's hematoxylin for 4 min, dehydrated, cleared, and mounted at room temperature.

The immunostained slides were independently examined by two pathologists. The evaluation was performed twice, and the pathologists were blinded to the clinicopathological features of the patients, including the specific diagnosis and prognosis of each individual patient. PD-1 positivity was defined as staining of >40% of T cells in a high-power field light microscope (magnification, x400) at the centre of the tumor. CD3 antibody (dilution 1:100; cat. no. ab5690; Abcam) was used to identify the intratumoral lymphocytes in immunohistochemistry staining. A biotinylated goat anti-rabbit antibody (dilution 1:100; cat. no. BA-1000; Vector Laboratories, Inc.) was used as the secondary antibody. PD-1 uptake in CD3-positive cells were defined as PD-1 positive. PD-L1-positive expression was defined by a cytoplasmic staining pattern in the tumor tissues. The PD-L1 staining intensity was graded as follows: i) 0, no staining; ii) 1, weak staining; iii) 2, moderate staining; and iv) 3, strong staining. Tumors with moderate or intense staining were classified as positive and tumors with no or weak staining as negative.

Preparation of peripheral blood mononuclear cells and tumor infiltrating lymphocytes. Peripheral blood samples (30 ml) were drawn from patients before surgery and centrifuged at 1,800 x g for 3 min through a Ficoll-Paque (GE Healthcare) gradient to isolate peripheral blood mononuclear cells (PBMCs). Freshly excised tumor tissues were homogenized and digested with 1.5 mg/ml collagenase D (Sigma-Aldrich; Merck KGaA). The resulting cell suspensions were filtered through a mesh filter (BD Biosciences). Due to the large amount of tissue required to isolate sufficient tumor infiltrating lymphocytes (TILs) for flow cytometry, TILs were obtained from 30 aforementioned additional patients.

Flow cytometry. Fluorescence-activated cell sorting (FACS) analysis was performed using a Navios flow cytometer (Beckman Coulter, Inc.) running Navios Platform 3.0 software (Beckman Coulter, Inc.). The following antibodies were used to classify cells: Anti-CD3-FITC (dilution 1:100; cat. no. 9515-02; SouthernBiotech), anti-CD4 phycoerythrin (PE; dilution 1:100; cat. no. 9522-09; SouthernBiotech), anti-CD8 PC5 (dilution 1:100; cat. no. 9536-16; SouthernBiotech), anti-PD-1 PC7

Table I. Isotype control antibodies.

Antibody	Model number	Manufacturer	City	Country
CD3 FITC	9515-02	SouthernBiotech	Birmingham, AL	USA
CD4 PE	9522-09	SouthernBiotech	Birmingham, AL	USA
CD8 PC5	9536-16	SouthernBiotech	Birmingham, AL	USA
PD1 PC7	25-2799-42	ebioscience; Thermo Fisher Scientific, Inc.	Waltham, Massachusetts	USA
PDL1 PC7	558017	BD Biosciences	San Jose, CA	USA
IgG1 FITC	0102-02	SouthernBiotech	Birmingham, AL	USA
IgG1 PE	0102-09	SouthernBiotech	Birmingham, AL	USA
IgG1 PC5	0102-16	SouthernBiotech	Birmingham, AL	USA
IgG1 PC7	25-4714-42	ebioscience; Thermo Fisher Scientific, Inc.	Waltham, Massachusetts	USA

PE, phycoerythrin; PC, phycoerythrin-cyanine.

(dilution 1:200; cat. no. 25-2799-42; ebioscience; Thermo Fisher Scientific, Inc.), anti-PD-L1 PC7 (dilution 1:200; cat. no. 558017; BD Biosciences), anti-IgG1 FITC (dilution 1:100, cat. no. 0102-02; SouthernBiotech), anti-IgG1 PE (dilution 1:100; cat. no. 0102-09; SouthernBiotech), anti-IgG1 PC5 (cat. no. 0102-16; SouthernBiotech,), and anti-IgG1 PC7 (dilution 1:100; cat. no. 25-4714-42; ebioscience; Thermo Fisher Scientific, Inc.). The gating strategy is provided in Fig. S1. The present study used isotype control antibodies for discriminating non-specific background staining (Table I).

Statistical analysis. Continuous variables are expressed as the mean ± SD. Two-sided P-values were determined by χ^2 test. The Kaplan-Meier method was used to estimate OS and DFS time. Cox regression multivariate models were used to identify independent prognostic factors. In order to perform Cox multivariate analysis, binary variable is required, therefore, the patients were divided into two groups: i) <60 years old; and ii) >60 years old (22). Unpaired Student's t-tests and χ^2 tests were used to compare the frequency of TILs among the subgroups. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demographic data. The clinicopathological data of the patients analyzed in the present study are presented in Table II. Out of 170 patients, 103 (60.6%) were male and 67 (39.4%) were female, with a mean age of 67.7±11.9 years (range, 27-85 years). A total of 27 (15.9%) patients had TNM stage I gastric adenocarcinoma, 56 (32.9%) had stage II, 75 (44.1%) had stage III, and 12 (7.0%) had stage IV.

PD-1 and PD-L1 expression in gastric cancer tissue and clinicopathologic features. Images of immunohistochemical staining for PD-1 and PD-L1 are shown in Fig. 1. PD-1 and PD-L1 were expressed in 30.0 and 60.5% of the gastric cancer tissues, respectively. The association between PD-1 and PD-L1 expression levels and the clinicopathological variables are presented in Table III. The expression of PD-1 was significantly higher in patients with perineural invasion (P=0.015). The expression of PD-L1 was significantly higher in patients with advanced T stage (P=0.035) or advanced TNM stage (P=0.050).

Effect of PD-1 and PD-L1 expression on survival. Analyses of survival, according to PD-1 and PD-L1 expression, are presented in Figs. 2 and 3. The patients positive for PD-L1 expression had a shorter DFS time compared with those negative for PD-L1 expression (P=0.005). Multivariate analysis using the Cox proportional hazards model identified venous invasion and TNM stage as independent prognostic factors associated with OS and DFS time in patients with gastric cancer (Table IV). In addition, PD-L1 expression was significantly associated with patient prognosis (P=0.015).

PD-1 and PD-L1 expression on circulating CD4+ and CD8+ T cells and clinicopathologic features. To assess PD-1 expression on T cells, $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs by flow cytometry. Cells were not stimulated before detecting PD-L1 expression on T cells. The association between the expression level of PD-1 on T cells and the clinicopathological characteristics of patients with gastric cancer was subsequently analyzed. PD-1 expression on $CD8^+$ T cells was significantly associated with depth of invasion (39.4±2.9% vs. 25.7±1.7%; P<0.001; Fig. 4A). However, PD-1 expression on $CD4^+$ T cells was not associated with depth of invasion (19.2±3.17% vs. 15.3±2.6%; P=0.755; Fig. 4B). No significant associations were identified between the expression levels of these two proteins and histologic types, lymph node metastasis or TNM stages.

PD-1 and PD-L1 expression on CD4+ and CD8+ T cells in gastric cancer tissue. PD-1 and PD-L1 expression levels on $CD4^+$ T cells and $CD8^+$ T cells were subsequently determined from gastric cancer tissues. PD-1 expression was significantly higher on $CD8^+$ T cells compared with $CD4^+$ T cells (Fig. 5A). PD-L1 expression was also significantly higher on $CD8^+$ T cells compared with $CD4^+$ T cells (Fig. 5B).

Discussion

The host immune system can recognize and destroy cancer cells (23). Therefore, immune evasion of cancer cells may play

Table II. Baseline clinical characteristics of patients with gastric cancer.

Basic characteristics	Values
Mean age ± SD, years	67.7±11.9
Sex, n (%)	
Male	103 (60.6)
Female	67 (39.4)
Histologic type, n (%)	
Well-differentiated	7 (4.1)
Moderately-differentiated	77 (45.3)
Poorly-differentiated	83 (48.8)
Mucinous	3 (1.8)
Signet component, n (%)	
No	127 (74.7)
Yes	43 (25.3)
Lauren classification, n (%)	
Intestinal	81 (47.6)
Diffuse	66 (38.8)
Mixed	23 (13.6)
Lymphatic invasion, n (%)	
Present	108 (63.5)
Absent	62 (36.5)
Venous invasion, n (%)	
Present	41 (24.1)
Absent	129 (75.9)
Perineural invasion, n (%)	
Present	74 (43.5)
Absent	96 (56.5)
T stage, n (%)	
T1	17 (10.0)
T2-4	153 (90.0)
N stage, n (%)	
N0	53 (31.2)
N1-3	117 (68.8)
M stage, n (%)	
M0	139 (81.8)
M1	31 (18.2)
TNM stage, n (%)	
I	27 (15.9)
II-IV	143 (84.1)
Total cases	170

TNM, Tumor-Node-Metastasis.

a critical role in the development and progression of tumors (23). PD-L1, a member of the B7 family of immune-regulatory cell surface proteins, suppresses the cell-mediated immune response by interacting with its receptor, PD-1 (24). Overexpression of PD-L1 by tumor cells has been reported in various types of cancer, such as pancreatic cancer, esophageal cancer, breast cancer, and gastric cancer and impairs T cell-mediated antitumor immunity (4).

In the present study, the expression of PD-1 and PD-L1 was determined in a large series of gastric cancer tissue samples to examine the association of the expression levels of these factors with clinicopathological characteristics and survival rates. The present results showed that PD-1 and PD-L1 expression is upregulated in gastric cancer cells, in line with previous reports (19,25-28). In addition, the expression levels of PD-1 and PD-L1 have been reported to be significantly associated with several adverse prognostic factors (27-30). Sun *et al* (29) reported that high PD-L1 expression in tumors was associated with decreased survival rate and poor prognosis in gastric cancer. In the present study, PD-L1 expression was associated with a more advanced T stage and TNM stage, which is consistent with the results reported by Eto *et al* (27). However, these results are not in line with the results reported by Kim *et al* (19), who found that PD-L1 expression tended to increase with decreasing tumor stage. In the present study, PD-1 expression was higher in patients with perineural invasion, which is a marker of poor prognosis (16). Therefore, high expression of PD-1 and PD-L1 may be associated with aggressive behavior of gastric cancer.

Previous studies have assessed the association of survival outcomes with PD-1 and PD-L1 expression in gastric cancer (29,30). Kim *et al* (19) reported that PD-L1 expression was associated with improved OS and DFS. Eto *et al* (27) reported that patients with and without PD-1 expression had similar OS rates; however, DFS was significantly decreased in the PD-1-positive group than in the PD-1 negative group. PD-L1 expression did not influence OS or DFS. In the present study, PD-L1 expression was significantly associated with a poorer DFS, while PD-1 expression was not significantly associated with prognosis. The present results suggested that PD-L1 expression may be a prognostic marker in gastric cancer.

PD-1 and PD-L1 play important roles in the regulation of the immune system and maintenance of peripheral tolerance through T cell activation and tolerance induction (15). PD-1 is expressed on T cells in response to inflammatory stimuli, and tumor cells express PD-L1 to inhibit T cell mediated antitumor immunity, where PD-L1 binds to PD-1 on TILs (15,31). In the present study, PD-1 expression on $CD4^+$ and $CD8^+$ T cells was increased significantly in patients with advanced gastric cancer compared with patients with early gastric cancer, suggesting that PD-1 expression is associated with tumor progression. However, the prognostic significance of PD-1 expression on T cells is unclear due to the small sample size of patients with early gastric cancer included in the present study. Therefore, further investigation of the prognostic significance of PD-1 expression on T cells in patients with gastric cancer is required.

PD-1 is expressed on the surface of activated macrophages, T lymphocytes, B lymphocytes, natural killer cells and on some myeloid cells such as myeloid dendritic cells (32). PD-L1, also known as B7-H1/CD274, is a B7 family member expressed primarily by hematopoietic and parenchymal cells that regulates self-tolerance *in vivo* by binding to PD-1 on T lymphocytes (32). In a previous study, PDL-1 was identified on the tumor surface and on parenchymal or antigen presenting cells (28). In a recent study by Arrieta *et al* (33), PD-L1 expression was examined on circulating $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cytotoxic cells from patients with advanced non-small cell lung cancer, and PD-L1 receptor expression

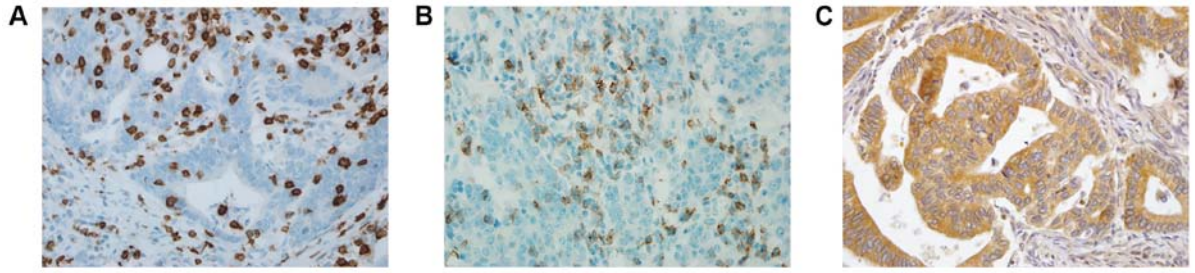

Figure 1. Immunohistochemical analysis of PD-1 and PD-L1 expression in gastric adenocarcinoma. Intratumoural lymphocytes are positive for (A) CD3 and (B) PD-1 (magnification, x400). (C) Gastric adenocarcinoma cells show diffuse cytoplasmic positivity for PD-L1 (magnification, x400). PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1.

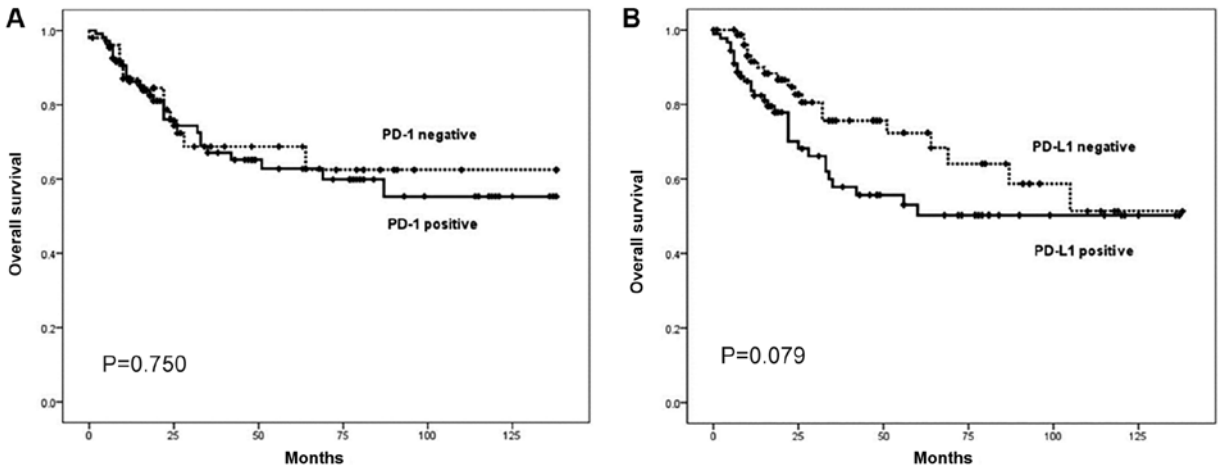

Figure 2. OS time with respect to PD-1 and PD-L1 expression. Kaplan-Meier analysis of OS time according to (A) PD-1 and (B) PD-L1 expression in patients with gastric carcinoma. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1; OS, overall survival.

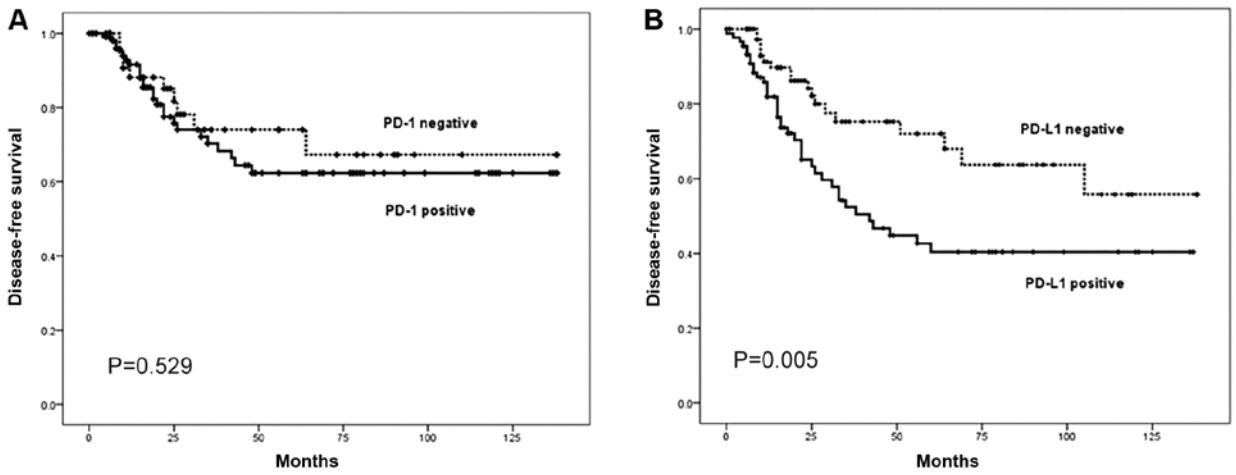

Figure 3. DFS time with respect to PD-1 and PD-L1 expression. Kaplan-Meier analysis of DFS time according to (A) PD-1 and (B) PD-L1 expression in patients with gastric carcinoma. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1; DFS, disease-free survival.

ratio was found to have lower percentages, 0.02-8.7% on $CD3^+CD4^+$ T and 0.08-8.78% on $CD3^+CD8^+$ cytotoxic T cells. Furthermore, Xu *et al* (34). reported that patients with colorectal cancer presented with significantly higher levels of circulating $Tim\text{-}3^+PD\text{-}1^+CD8^+$ T cells compared to the healthy controls (medians of 3.12 and 1.99%, respectively; P=0.04). However, Saito *et al* (26). reported that PD-1 expression on $CD4^+$ T cells obtained from PBMC, normal gastric mucosa, and gastric cancer tissue was 31.0±7.2, 59.5±10.6, and 73.4±9.9%, respectively. They also reported that the frequency of $PD\text{-}1^+$ $CD4^+$ T-cells from gastric cancer tissue with PD-L1 expression was significantly higher than that from gastric cancer tissue without PD-L1 expression (49.7±10.4% vs. 30.6±9.7%, respectively) (35). This study indicated high expression of

Table III. Association between expression of PD-1, PD-L1 and clinicopathological parameters.

	PD-1 expression			PD-L1 expression		
Variables	Negative	Positive	P-value	Negative	Positive	P-value
Mean age ± SD, years	62.9±12.7	62.5±11.4	0.636	63.5±12.4	62.1±11.7	0.456
Sex, n (%)			0.498			0.265
Male	70 (58.8)	33 (64.7)		37 (55.2)	66 (64.1)	
Female	49 (41.2)	18 (35.3)		30 (44.8)	37 (35.9)	
Histologic type, n (%)			0.253			0.035[a]
Well-differentiated	6 (5.0)	1 (2.0)		0 (0)	7 (6.8)	
Moderately-differentiated	49 (41.2)	28 (54.9)		27 (41.9)	50 (48.5)	
Poorly-differentiated	61 (51.3)	22 (43.1)		37 (55.6)	45 (44.6)	
Mucinous	3 (2.5)	0 (0)		2 (2.5)	1 (1.9)	
Signet component, n (%)			0.178			0.475
No	85 (71.4)	42 (82.4)		48 (71.6)	79 (76.7)	
Yes	34 (28.6)	9 (17.6)		19 (28.4)	24 (23.3)	
Lauren classification, n (%)			0.707			0.39
Intestinal	54 (72.9)	27 (52.9)		27 (40.3)	54 (52.4)	
Diffuse	49 (80.4)	17 (33.3)		30 (44.8)	36 (34.9)	
Mixed	16 (61.5)	7 (13.8)		10 (14.9)	13 (12.7)	
Lymphatic invasion, n (%)			0.487			0.258
Absent	41 (34.5)	21 (41.2)		28 (41.8)	34 (33.0)	
Present	78 (65.5)	30 (58.8)		39 (58.2)	69 (67.0)	
Venous invasion, n (%)			0.784			0.583
Absent	91 (76.5)	38 (74.5)		49 (73.1)	80 (77.7)	
Present	28 (23.5)	13 (25.5)		18 (26.9)	23 (22.3)	
Perineural invasion, n (%)			0.015[a]			0.115
Absent	99 (83.1)	15 (39.4)		43 (64.2)	53 (51.5)	
Present	20 (16.9)	36 (70.6)		24 (35.8)	50 (48.5)	
T stage, n (%)			0.289			0.035[a]
T1	10 (8.4)	7 (13.7)		11 (16.4)	6 (5.8)	
T2-4	109 (91.6)	44 (86.3)		56 (83.6)	97 (94.2)	
N stage, n (%)			0.943			0.401
N0	38 (31.9)	16 (31.4)		24 (35.8)	30 (29.1)	
N1-3	81 (68.1)	35 (68.6)		43 (64.2)	73 (70.9)	
M stage, n (%)			0.153			0.93
M0	94 (79.0)	45 (88.2)		55 (82.1)	84 (81.6)	
M1	25 (21.0)	6 (11.8)		12 (17.9)	19 (18.4)	
TNM stage, n (%)			0.137			0.05
I	16 (13.4)	11 (21.6)		15 (22.4)	12 (11.7)	
II-IV	103 (86.6)	40 (78.4)		52 (77.6)	91 (88.3)	

[a]P<0.05. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1; TNM, Tumor-Node-Metastasis.

PDL-1 on $CD4^+$ and $CD8^+$ T cells, which was in accordance with Saito *et al* (26,35). The distinction between the data of the present study and the data by Saito *et al* (26,35) compared with Arrieta *et al* (33) and Xu *et al* (34) are probably due to the tumors in different organs.

Thompson *et al* (28) reported that $CD8^+$ T cell infiltration in tumors and at peritumoural interfaces was increased in PD-L1-positive compared with PD-L1-negative patients. In addition, 89% of stroma PD-L1 positive tumors had high CD8 densities, suggesting an association with $CD8^+$ T cells, which produce cytokines such as interferon γ and express PD-L1 (28). In a meta-analysis, Gu *et al* (36) demonstrated that patients with Epstein-Barr virus infection (EBV^+) and microsatellite instability (MSI) are more likely to express PD-L1. EBV^+ and MSI gastric

Table IV. Cox multivariate analysis of clinicopathological risk factors affecting survival rate of patients with gastric cancer.

	Overall survival rate		Disease-free survival rate	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value
Age, years		0.121		0.06
<60	1		1	
≥60	0.972 (0.939-1.007)		0.967 (0.933-1.001)	
Lymphatic invasion		0.996		0.613
Absent	1		1	
Present	0.997 (0.394-3.384)		1.344 (0.426-4.238)	
Venous invasion		0.012[a]		0.029[a]
Absent	1		1	
Present	3.663 (1.330-10.092)		3.143 (1.127-8.763)	
Perineural invasion				
Absent	1	0.208	1	0.087
Present	1.818 (0.718-4.607)		2.202 (0.893-5.429)	
TNM stage		0.05		0.008[a]
I	1		1	
II	1.160 (0.549-3.863)		1.998 (0.977-3.042)	
III	2.265 (1.066-5.727)		3.178 (1.872-9.372)	
IV	5.483 (3.399-11.372)		5.051 (1.753-11.372)	
PD-L1 expression		0.497		0.015[a]
Negative	1		1	
Positive	1.373 (0.550-3.426)		3.033 (1.237-7.440)	

[a]P<0.05. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1; TNM, Tumor-Node-Metastasis; HR, hazard ratio; CI, confidence interval.

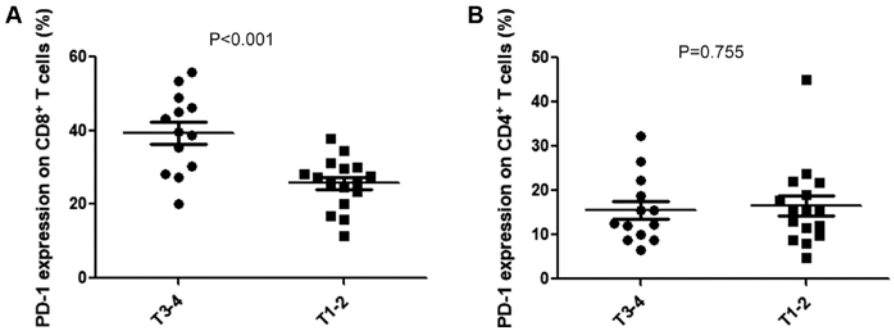

Figure 4. Association between PD-1 expression on CD8+ T cells, and CD4+ T cells and T stage. (A) PD-1 expression on CE8+ T cells is significantly associated with depth of invasion. (B) PD-1 expression on CD4+ T cells is not associated with depth of invasion. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1.

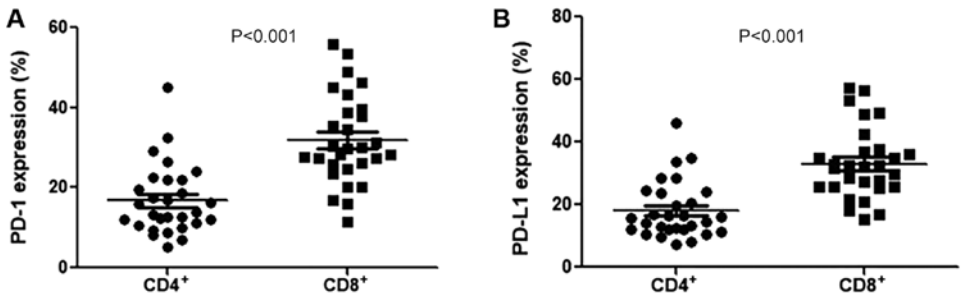

Figure 5. PD-1 and PD-L1 expression levels on CD4+ T cells and CD8+ T cells. (A) PD-1 expression is significantly higher on CD8+ T cells compared with CD4+ T cells. (B) PD-L1 expression is significantly higher in CD8+ T cells than in CD4+ T cells. PD-1, programmed death-1; PD-L1, programmed death-1 ligand-1.

cancer exhibits lymphocytic infiltration in tumor stroma; therefore, the lymphoid stroma in these tumors has a large number of CD8 T cells, which are capable of mounting a robust antitumor inflammatory response (36). In addition, PD-L1 expression is associated with a concomitant and significant increase in the number of CD8 T cells at the tumor invasive front (37). In the present study, PD-1 and PD-L1 expression levels were significantly higher on circulating $CD8^+$ T cells than on circulating $CD4^+$ T cells. The present results suggested evasion of the adaptive immune response in these tumors, which may be overcome by administration of anti-PD-1/PD-L1. However, the interaction between $CD8^+$ T cells and immune evasion, mediated by increased PD-1 and PD-L1 expression, requires further examination.

The present study presented several limitations. First, digital imaging analyses for the assessment of PD-1 and PD-L1 immunohistochemical outcomes could not be performed, which could be problematic for inter-observer variation. Second, a retrospective cohort study was conducted to evaluate the prognostic significance of PD-1 and PD-L1; however, the clinicopathological characteristics among the patients analyzed were heterogeneously distributed. Therefore, selection bias might have influenced the outcome. The finding that PD-L1 expression showed no effect on OS may be due to the small sample size. In addition, when, the patients were divided into two groups according to PD-L1 expression status, bias might have occurred due to the difference of distribution in Lauren classification and differentiation between the two groups. Third, the absence of data using five-color staining and analyzing the markers together is a limitation of this study. Fourth, there are some other cells, such as $CD4^+CD8^+$ and $CD4^-CD8^-$, that can also express PD-1 and PD-L1 (38) and these cells may also be important for the tumor progression. However, the expression of PD-1 and PD-L1 in the other cells, such as $CD4^+CD8^+$ and $CD4^-CD8^-$ was not examined in the present study.

In conclusion, upregulation of PD-1 on $CD4^+$ and $CD8^+$ T cells may play a role in the immune evasion of gastric cancer. Furthermore, PD-L1 expression may be an independent indicator of poor prognosis in patients with gastric cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

KHJ, SYK and EYS designed the study. JHK, JKJ, HJC and EYS performed the experiments. KHJ and SYK analyzed the data. KHJ and JHK wrote the manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Review Board (approval no. VC14SISI0230) at St. Vincent's Hospital, The Catholic University of Korea (Suwon, Republic of Korea). For tissue sample collection, the study protocol was approved by the Institutional Review Board (approval no. VC16TISI0196) at St. Vincent's Hospital, The Catholic University of Korea (Suwon, Republic of Korea). The patients whose tissues were used for flow cytometry provided written informed consent.

Patient consent for publication

Patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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