ANTICANCER RESEARCH *38*: 107-112 (2018)
doi:10.21873/anticanres.12197

Highly Activated PD-1/PD-L1 Pathway in Gastric Cancer with PD-L1 Expression

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Abstract. *Background: A recent study demonstrated that immune-checkpoint molecules are associated with tumoral immune evasion. Materials and Methods: Programmed cell death protein 1 (PD-1) expression on $CD4^+$ and $CD8^+$ T-cells obtained from gastric cancer tissue was evaluated by multicolor flow cytometry. Immunohistochemical staining was also performed to evaluate programmed cell death ligand-1 (PD-L1) expression on gastric cancer cells. Results: There were statistically significant correlations between PD-L1 expression and age, histology, tumor size, depth of invasion, lymph node metastasis, lymphatic vessel invasion, venous invasion, and disease stage. The 5-year survival rates of patients with and without PD-L1–positive tumors were 48.9% and 80.7%, respectively, and the difference was statistically significant. Multivariate analysis indicated that PD-L1 expression was an independent prognostic indicator. The frequency of PD-1-positive $CD4^+$ and $CD8^+$ T-cells from gastric cancer tissue with PD-L1 expression was significantly more than that from gastric cancer tissue without PD-L1 expression. Conclusion: PD-L1 expression was related to a poor prognosis in patients with gastric cancer. Furthermore, PD-1 expression on T-cells was up-regulated in patients with tumors with PD-L1 expression.*

Gastric cancer is one of the most common malignancies. Although the prognosis of gastric carcinoma has improved because of the greater availability of diagnostic techniques and more effective intraoperative and postoperative care, gastric cancer still ranks second among all cancer-related deaths worldwide (1).

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Key Words: Gastric cancer, PD-1, PD-L1, T-cells.

Cancer immunotherapy is now receiving considerable attention because of the success of immune-checkpoint inhibitors [*e.g.* antibodies to programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4)] in the treatment of various tumor types (2-4). These successes indicate that effective immunity to cancer cells can be induced even in patients with cancer. Many cancer cells express tumor antigens that render them susceptible to recognition and lysis by T-cells (5). However, spontaneous rejection of established tumors is rare because cancer cells frequently use physiological immunosuppressive mechanisms to escape from host immunity, a phenomenon known as tumoral immune evasion. Although the detailed mechanisms responsible for tumoral immune evasion remains unclear, some reported mechanisms include the secretion of immunosuppressive cytokines and the presence of immune-regulatory cells, including regulatory T-cells (6) and myeloid-derived suppressor cells (7). A recent study demonstrated that immune-checkpoint molecules also seem to be associated with tumoral immune evasion. In this regard, programmed cell death ligand-1 (PD-L1) overexpression has been demonstrated in various types of cancer (8-13). PD-L1 is the ligand for PD-1, a well-known immune-checkpoint molecule (8, 14). PD-L1 is usually expressed in antigen-presenting cells, such as dendritic cells, macrophages, and monocytes. PD-1/PD-L1 interactions contribute to the maintenance of peripheral tolerance of self-antigens in normal hosts (9). Mounting evidence suggests that PD-L1 expression on solid tumors dampens antitumor T-cell responses (8-13). Blockage of PD-L1 inhibited tumor growth or delayed tumor progression in multiple murine models (12, 13, 15, 16), and adoptive transfer of tumor-specific $PD\text{-}1^{-/-}$ T-cell receptor transgenic T-cells rejected tumors even when $CTLA\text{-}4^{-/-}$ transgenic T-cells were unable to (10). Moreover, PD-L1 tumor expression has been shown to correlate with an inferior clinical outcome in various solid human malignancies (17-21). In contrast, however, there are conflicting reports with regard to the correlation between PD-L1 expression and prognosis in patients with gastric cancer. Eto *et al.* reported that PD-L1 expression was an

independent prognostic factor after curative resection of gastric cancer (22). Conversely, Kawazoe *et al*. reported that PD-L1 expression was not associated with a poor prognosis in patients with gastric cancer (23). Chang *et al*. demonstrated that PD-L1 expression in gastric adenocarcinoma was a poor prognostic factor among patients with high levels of $CD8^+$ tumor-infiltrating lymphocytes (24), indicating the possibility that the effect of PD-L1 on prognosis depends on the status of lymphocytes in patients with gastric cancer. PD-L1 delivers a negative signal to lymphocytes by binding to PD-1 expressed on lymphocytes, thus inducing functional impairment of the lymphocytes. PD-1 expression on lymphocytes is also likely to be related to the effect of PD-L1 on prognosis. However, no reports have shown a correlation between PD-L1 expression on cancer cells and PD-1 expression on lymphocytes. In the current study, therefore, we investigated the correlation between PD-L1 expression and prognosis in patients with gastric cancer. Furthermore, we determined the correlation between PD-L1 expression on cancer cells and PD-1 expression on lymphocytes.

Materials and Methods

Patients and normal donors. In total, 157 patients pathologically diagnosed with gastric adenocarcinoma and treated at Tottori University Hospital were enrolled in the present study. The study protocol was approved by the Institutional Review Board at Tottori University Hospital (Yonago, Japan; Approval number 1705A040). None of the patients received radiotherapy, chemotherapy, or other medical interventions before surgery. The clinicopathological findings were determined according to the Japanese Classification of Gastric Carcinoma (25).

Immunohistochemistry. Tissue samples were fixed in formalin and embedded in paraffin. Serial sections were cut at 4 μm, dewaxed, deparaffinized in xylene, and rehydrated through a graded alcohol series. For retrieval of PD-L1, the sections were boiled for 15 min in a microwave oven in citrate buffer (pH 9.0). The samples were incubated in 3% hydrogen peroxidase for 10 min to block endogenous peroxidases and in Block Ace (DS Pharma Biomedical, Osaka, Japan) for 20 min to prevent nonspecific antigen binding. The slides were subsequently incubated with rabbit polyclonal anti-PD-L1 (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Secondary antibody binding was detected with Histofine Simple Stain MAX-PO (Nichirei, Tokyo, Japan), and the sections were developed with Histofine DAB Solution (Nichirei) and counterstained with Mayer's hematoxylin. The presence of cells positive for PD-L1 on each slide was determined in a blinded manner. Five high-power fields were randomly selected, and the percentage of positive cells in these fields was counted. Patterns with ≥5% positive cells were considered positive, and those with <5% positive cells were considered negative, as described previously (26).

Flow cytometric analysis. Freshly excised tumor tissues were minced and digested with 1.5 mg/ml of collagenase D (Wako Pure Chemical Industries Ltd., Osaka, Japan). The resulting cell suspensions were filtered through a mesh filter (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were passed through a mesh filter and used for fluorescence-activated cell sorting analysis performed using a FACSCalibur™ (Becton Dickinson). The following antibodies were used to classify cells: anti-CD3-phycoerythrin (PE)-Cy5 (BioLegend, San Diego, CA, USA), anti-CD4-fluorescein (FITC), anti-CD8-FITC, and anti-PD-1-PE (all from Becton Dickinson).

Statistical analysis. Between-group differences were analyzed by the Mann–Whitney *U*-test. Disease-specific survival was calculated according to the Kaplan–Meier method and compared using the log-rank test. Patients who died of causes other than gastric cancer were considered lost to follow-up at the time of death. Multivariate analysis of factors prognostic for disease-specific survival was performed using a Cox proportional hazards model and a stepwise procedure. Statistical significance was defined as $p<0.05$. Statistical analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and StatView 5.0 for Windows (SAS Institute, Cary, NC, USA) software.

Results

PD-L1 expression in gastric cancer tissue and clinicopathological characteristics. The expression of PD-L1 was evaluated by immunohistochemical staining and was mainly located on the membrane and in the cytoplasm of gastric cancer cells (Figure 1). Among the 157 patients included in the current study, 41 patients (26.8%) were considered to be PD-L1–positive. Table I shows the correlation between the patient clinicopathological characteristics and PD-L1 expression. Patients with PD-L1-positive tumors were significantly older than those with PD-L1-negative tumors ($p=0.019$). Undifferentiated adenocarcinoma was observed more frequently in patients with PD-L1-positive tumors than in those with PD-L1-negative tumors. Furthermore, there were statistically significant correlations between PD-L1 expression and tumor size ($p=0.003$), depth of invasion ($p<0.0001$), lymph node metastasis ($p<0.0001$), lymphatic vessel invasion ($p<0.0001$), venous invasion ($p=0.0003$), and disease stage ($p<0.0001$).

PD-L1 expression and prognosis. Among 153 patients who underwent R0 resection, the 5-year survival rates of patients with and those without PD-L1-positive tumors were 48.9% and 80.7%, respectively ($p<0.0001$) (Figure 2). Furthermore, the multivariate analysis indicated that PD-L1 expression, depth of invasion, and blood vessel invasion were independent prognostic indicators (Table II).

Correlation between PD-L1 expression on gastric cancer cells and PD-1 expression on tumor-infiltrating lymphocytes. We then evaluated PD-1 expression on $CD4^+$ and $CD8^+$ T-cells isolated from gastric cancer tissues by flow cytometry. The frequency of PD-1-positive $CD4^+$ T-cells from gastric cancer tissue with PD-L1 expression was significantly more than that from gastric cancer tissue without PD-L1 expression (49.7%±10.4% *versus* 30.6%±9.7%, respectively; $p<0.0001$) (Figure 3). The frequency

Table I. *Programmed cell death ligand-1 (PD-L1) expression and clinicopathologic characteristics*

	PD-L1		
	Negative (n=115)	Positive (n=42)	*p*-Value
Age, years	66.4±11.0	71.2±13.0	0.019
Gender, n (%)			
Male	88 (76.5%)	32 (76.2%)	0.97
Female	27 (23.5%)	10 (23.8%)	
Tumor size, (cm)	4.1±3.0	5.2±2.7	0.003
Histology, n (%)[a]			0.03
Differentiated	58 (50.4%)	13 (31.0%)	
Undifferentiated	57 (49.6%)	29 (69.0%)	
Depth of invasion, n (%)			<0.0001
Early	67 (58.3%)	7 (16.7%)	
Advanced	48 (41.7%)	35 (83.3%)	
Lymph node metastasis, n (%)		<0.0001	
Absent	79 (68.7%)	9 (21.4%)	
Present	36 (31.3%)	33 (78.6%)	
Lymphatic vessel invasion, n (%)		<0.0001	
Absent	45 (39.1%)	2 (4.8%)	
Present	70 (60.9%)	40 (95.2%)	
Blood vessel invasion, n (%)		0.0003	
Absent	53 (46.1%)	6 (14.3%)	
Present	62 (53.9%)	36 (85.7%)	
Stage of disease, n (%)			<0.0001
I/II	93 (80.9%)	19 (45.2%)	
III/IV	22 (19.1%)	23 (54.8%)	

[a]Differentiated, papillary, or tubular adenocarcinoma; undifferentiated, poorly differentiated, mucinous adenocarcinoma, and signet-ring cell carcinoma.

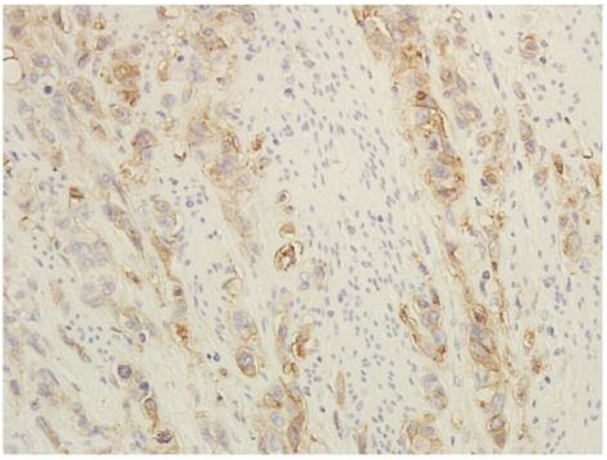

Figure 1. *Representative image of positive programmed cell death ligand-1 expression in gastric cancer tissue (magnification ×400).*

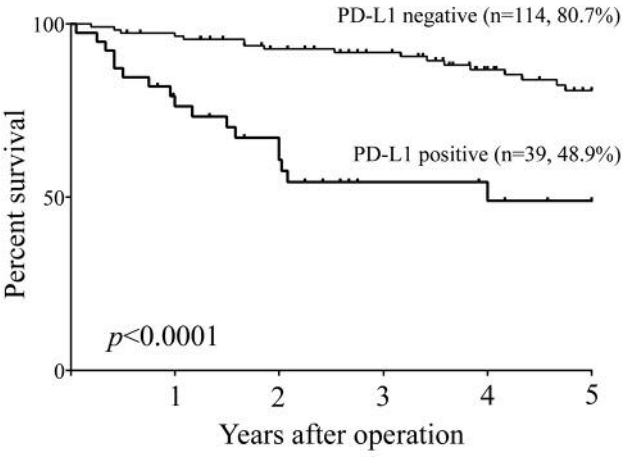

Figure 2. *Prognosis of patients with gastric cancer according to programmed cell death ligand-1 (PD-L1) expression. The prognosis of patients with PD-L1-positive gastric cancer was significantly worse than that of patients with PD-L1-negative tumors.*

of PD-1-positive CD8+ T-cells from gastric cancer tissue with PD-L1 expression was also significantly more than that from gastric cancer tissue without PD-L1 expression (42.5%±14.0% *versus* 30.7%±14.7%, respectively; p=0.033) (Figure 4).

Discussion

PD-L1 is usually expressed on antigen-presenting cells, such as dendritic cells, macrophages, and monocytes. Importantly, PD-L1 expression on monocyte-derived myeloid dendritic cells from both peripheral blood and cancer tissue is up-regulated and associated with immune suppression in patients with ovarian cancer (11). In this regard, we previously demonstrated that tumor-associated macrophages (TAMs) up-regulated PD-L1 expression in gastric cancer tissue (27), indicating the possibility that PD-L1 expression on TAMs is also associated with immune suppression in gastric cancer. Other studies have demonstrated that PD-L1 is also expressed on a wide variety of tumors and is a component of the immunosuppressive milieu (28, 29). Overexpression of PD-L1 in gastric cancer cells was also shown in the current study, indicating the presence of abundant PD-L1-expressing cells in gastric cancer tissue. It is likely that this phenomenon plays an important role in the escape of tumor cells from host immune surveillance, enabling tumor cell progression and metastasis to distant organs because PD-1/PD-L1 interaction is strongly associated with T-cell dysfunction. In fact, PD-L1 expression was observed more frequently in advanced stage of the disease. The present results indicate that PD-L1 expression is significantly associated with a poor prognosis in patients with gastric cancer. Furthermore, PD-L1 expression was an independent prognostic indicator. These results indicate the importance of PD-L1 in the progression of gastric cancer.

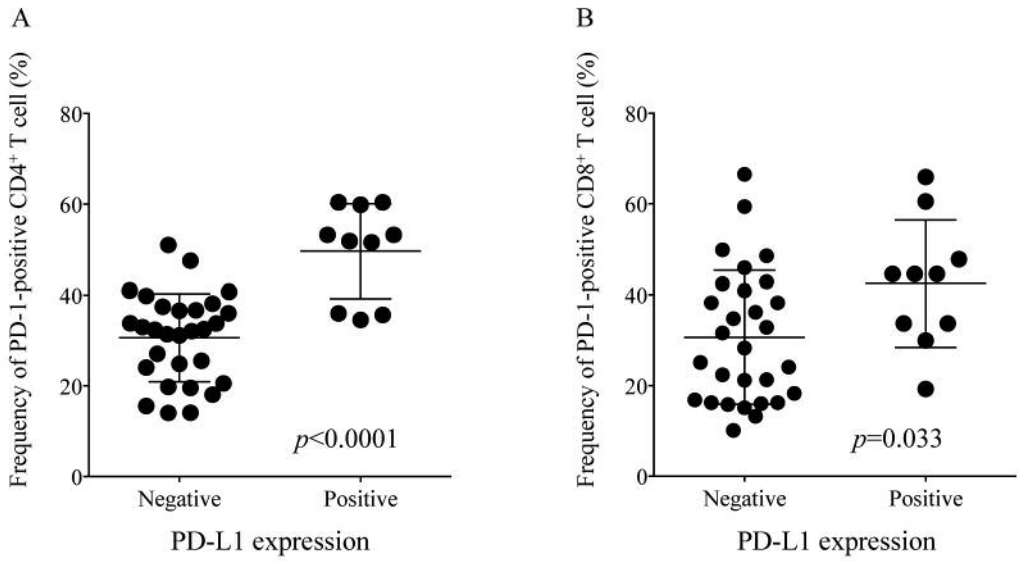

Figure 3. *Programmed cell death protein 1 (PD-1) expression on CD4+ (A) and CD8+ (B) T-cells according to programmed cell death ligand-1 (PD-L1) expression on gastric cancer tissues. The frequency of PD-1-positive CD4+ T-cells obtained from PD-L1-positive gastric cancer tissue was significantly more than that from PD-L1-negative gastric cancer tissue. The frequency of PD-1-positive CD8+ T-cells obtained from PD-L1-positive gastric cancer tissue was significantly more than that from PD-L1-negative gastric cancer tissue.*

Although tumor cells and TAMs overexpress PD-L1, these cells are unable to deliver a negative signal to T-cells without binding to the PD-1 expressed on T-cells. PD-1 expression is usually observed in activated T-cells. In acute infections, PD-1 is upregulated upon T cell activation but declines with resolution of the infection and establishment of memory (30). In contrast, exhausted antigen-specific CD8+ T-cells have been observed in chronic human immunodeficiency virus and hepatitis B and C virus infections in humans, as well as Simian immunodeficiency virus infection in monkeys. Recent work has shown that these exhausted viral-specific T-cells express high levels of PD-1 and that blockade of the PD-1 pathway can enhance in vitro T-cell responses (31), indicating that PD-1 expressed on T-cells plays an important role in exhausting T-cells in chronic viral infections. Interestingly, like chronic infections, tumors may also exploit the PD-1 pathway to evade eradication by the immune system. In fact, Matsuzaki *et al*. recently demonstrated that tumor-infiltrating NY-ESO-1-specific $CD8^+$ T-cells up-regulated PD-1 expression, which resulted in suppression of those $CD8^+$ T-cells (32). Furthermore, we previously demonstrated that both $CD4^+$ and $CD8^+$ T-cells up-regulated PD-1 expression in both the circulation and gastric cancer tissue in patients with gastric cancer (33). We also demonstrated that PD-1 expression was significantly higher in T-cells obtained from gastric cancer tissue than circulating T-cells, indicating the possibility that the tumor microenvironment induces PD-1 expression in patients with gastric cancer (33). Therefore, we determined the correlation between PD-L1 expression on tumor cells and PD-1 expression on T-cells obtained from gastric cancer tissue and found that the frequency of PD-1-positive $CD4^+$ and $CD8^+$ T-cells obtained from gastric cancer tissue with PD-L1 expression was significantly more than that from gastric cancer tissue without PD-L1 expression, indicating the close correlation between PD-L1 expression on tumor cells and PD-1 expression on T-cells in the tumor microenvironment. This result indicates the possibility that the same factor might induce up-regulation of both PD-L1 and PD-1. With regard to factors associated with up-regulation of PD-L1, Abiko *et al*. reported that interferon-gamma was responsible for the up-regulation of PD-L1 expression on cancer cells and TAMs (34). Previous reports have demonstrated that up-regulation of PD-1 on $CD8^+$ T-cells is related to interleukin (IL)-10 and IL-6, but not

Table II. *Multivariate analyses of prognostic factors for disease-specific survival in patients with gastric cancer.*

	HR	95% CI	*p*-Value
Factor			
Depth of invasion (T: 1, 2, 3, 4)[a]	2.277	1.222-4.241	0.0095
Blood vessel invasion (v: 0, 1, 2, 3)[b]	2.161	1.322-3.532	0.0021
PD-L1 (positive *vs*. negative)	2.451	1.019-5.882	0.045

HR: Hazard ratio; CI: confidence interval. [a]T1, Tumor invasion of the *lamina propria* or submucosa; T2, tumor invasion of the *muscularis propria*; T3, tumor invaded the subserosa; T4, tumor invasion is contiguous with or extends beyond the serosa or the tumor invades adjacent structures. [b]Blood vessel invasion: v0-v3, grade of venous invasion.

transforming growth factor-beta. We previously reported that the serum IL-6 concentration was significantly higher in patients with gastric cancer than in healthy controls (35). In addition, we also demonstrated that gastric cancer cells produce IL-10 (36). Previous reports have also demonstrated that PD-L1 up-regulation is related to soluble factors, such as IL-6 and IL-10 (37, 38). Therefore, these cytokines may be responsible for the up-regulation of both PD-1 and PD-L1 observed in the current study. Further investigations to clarify the mechanisms responsible for PD-1 and PD-L1 up-regulation in gastric cancer are urgently required.

The efficacy of the anti-PD-1 antibodies nivolumab and pembrolizumab for the treatment of cancer has been demonstrated in previous studies (3, 4). A recent study also indicated that pembrolizumab was effective for the treatment of gastric cancer (39). Furthermore, anti-PD-L1 for the treatment of cancer is under investigation. Considering the up-regulation of both PD-1 and PD-L1 in the tumor microenvironment of gastric cancer, it is highly expected that antibody to PD-L1 will also be effective for the treatment of gastric cancer. Conversely, our data suggest the presence of some factors that upregulated both PD-1 and PD-L1 in the tumor microenvironment of gastric cancer. These factors might be the next targets with which to develop more effective treatments for gastric cancer. Immunotherapy that targets factors responsible for up-regulation of PD-1 and PD-L1 might provide a breakthrough in the treatment of gastric cancer.

In conclusion, PD-L1 expression was related to a poor prognosis in patients with gastric cancer. Furthermore, PD-1 expression on T-cells was up-regulated in tumors with PD-L1 expression. Therefore, immunotherapy that targets factors responsible for the up-regulation of PD-1 and PD-L1 might provide a breakthrough in the treatment of gastric cancer.

Human Rights Statement and Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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Received October 23, 2017
Revised November 7, 2017
Accepted November 10, 2017