Clinical Significance of Programmed Death-1 Ligand-1 and Programmed Death-1 Ligand-2 Expression in Human Esophageal Cancer

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Abstract **Purpose:** The negative regulatory programmed death-1/programmed death-1 ligand (PD-1/PD-L) pathway in T-cell activation has been suggested to play an important role in tumor evasion from host immunity. In this study, we investigated the expression of PD-L1 and PD-L2 in human esophageal cancer to define their clinical significance in patients' prognosis after surgery.

Experimental Design: *PD-L1* and *PD-L2* gene expression was evaluated in 41 esophagectomy patients by real-time quantitative PCR. The protein expression was also evaluated with newly generated monoclonal antibodies that recognize human PD-L1 (MIH1) and PD-L2 (MIH18).

Results: The protein and the mRNA levels of determination by immunohistochemistry and real-time quantitative PCR were closely correlated. PD-L – positive patients had a significantly poorer prognosis than the negative patients. This was more pronounced in the advanced stage of tumor than in the early stage. Furthermore, multivariate analysis indicated that PD-L status was an independent prognostic factor. Although there was no significant correlation between PD-L1 expression and tumor-infiltrating T lymphocytes, PD-L2 expression was inversely correlated with tumor-infiltrating $CD8^+$ T cells.

Conclusions: These data suggest that PD-L1 and PD-L2 status may be a new predictor of prognosis for patients with esophageal cancer and provide the rationale for developing novel immunotherapy of targeting PD-1/PD-L pathway.

Esophageal cancer is the sixth leading cause of cancer-related death worldwide and one of most difficult gastrointestinal tumors to treat and cure (1). Systematic metastasis is present in more than 50% of patients at the time of the diagnosis. Surgery is standard treatment for localized and resectable esophageal cancer and surgeons in many countries have challenged this fatal disease with extended surgery for a last few decades (2, 3). Regardless of these efforts, patients often experience distant metastasis or local recurrence even after curative operation. Further attempts to combine preoperative chemotherapy and/or radiotherapy with surgery have often failed to show significant survival benefit (4 – 6). Consequently, long-term outcome is still unfavorable and latest data have reported that overall 5-year survival after surgery is only 25% to 40%. Thus, to improve patients' prognosis, novel strategies need to be developed and established.

Programmed death-1 (PD-1) is a costimulatory molecule that provides an inhibitory signal in T-cell activation. PD-l belongs to the CD28 family, and its extracellular region is 28% identical to CTLA-4 (7, 8). PD-1 is expressed on T cells, B cells, and myeloid cells. Two ligands for PD-1, PD-L1 (B7-H1) and PD-L2 (B7-DC), have been identified and those are cell-surface glycoprotein belonging to the B7 family (9 – 12). Although these two molecules share 34% identity of amino acid, their expression has been suggested to be differentially regulated (13, 14). Previous studies have shown that PD-1/PD-L interaction inhibits T-cell growth and cytokine secretion (10, 12). In addition, direct evidence that PD-1 – deficient mice develop spontaneous autoimmune diseases further suggests an inhibitory and regulatory role for PD-1/PD-L interaction in T-cell responses and the maintenance of self-tolerance (8, 15). Besides these fundamental immunologic roles of PD-L molecule, recent studies suggested the potential role of PD-L in tumor immunity (16). In tumors, cancer cell – associated PD-L1 increases apoptosis of antigen-specific T-cell clones *in vitro* (16). Furthermore, PD-L1 blockade using anti-PD-L1 monoclonal antibody enhanced antitumor immunity and inhibited tumor growth *in vivo* (17). Therefore, PD-L1 has been suggested to play an important role in the immune evasion from host immune system. Although these studies have been well shown

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either *in vitro* or *in vivo* using murine tumor models, the clinical relevance remains unknown (16, 17). In addition, PD-L1 expression has been reported in human carcinoma of lung, ovary, and colon and in melanomas, however, its effect on patient's prognosis and characteristics has not been determined yet (16). On the other hand, the function of PD-L2 in tumors remains largely unknown and only a few recent reports have suggested that PD-L2 may also play some roles in tumor immunity (18, 19). Liu et al. (18) showed that PD-L2 on the tumor cells promotes CD8 T-cell–mediated rejection at both the induction and effector phase of antitumor immunity. However, there is little information of PD-L2 expression in clinical tumors and its clinical relevance. Therefore, we investigated the PD-L1 and PD-L2 expression in human esophageal cancer to define the clinical significance. We found that the expression of either PD-L1 or PD-L2 is a significant prognostic marker in postoperative esophageal cancer patients.

Materials and Methods

Patients. We examined 41 patients with esophageal cancer who underwent surgery at Department of Surgery, Nara Medical University, between November 1995 and July 2002. The median age of the patients was 63 years, with a range of 46 to 73 years. When distant metastasis was solitary and resectable, subtotal esophagectomy was done with the combined resection of the metastatic tumor. Postoperative pathohistologic analysis indicated that all tumors evaluated in this study were squamous cell carcinoma. Tissues were obtained from the resected specimens and then were rapidly frozen at −80°C for storage until use. For immunohistochemistry, a part of fresh tumor tissue specimen was immediately embedded in optimum cutting temperature compound (Miles, Kankakee, IL), and frozen sections were then cut on the cryostat to thickness of 5 μm. The remainder of each specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. A serial section from each specimen was stained with H&E for histologic evaluation. Tumors were classified according to the tumor-node-metastasis staging system (20). The median follow-up for all patients was 25 months, with a range of 1 to 66 months.

Analysis of mRNA expression level. Total RNA was isolated using the guanidine isothiocyanate method (RNeasy Protect Starter Kit, RNeasy Mini Kit, Qiagen, Tokyo, Japan) and was transcribed to cDNA using cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the protocol of the manufacturer. Real-time quantitative PCR analysis was done by using ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). All primer/probe sets for PD-L1, PD-L2, $CD4^+$, and $CD8^+$ T cells were purchased from PE Applied Biosystems. PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 1 μL of cDNA in a 20-μL final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. The expression level of the housekeeping gene β_2-microglobulin was measured as an internal reference with a standard curve to determine the integrity of template RNA for all of the specimens. The ratio of mRNA level of each gene was calculated as follows: (absolute copy number of each gene) / (absolute copy number of β_2-microglobulin). We set several cutoff points arbitrarily to select the best value preliminarily with respect to postoperative prognosis. The most significant *P* value for the survival was found by a relative cutoff point for positive PD-L1 expression of 1.0 and positive PD-L2 expression of 38.0. When the expression ratio of a given specimen for PD-L1 was >1.0, it was considered to indicate positive *PD-L1* gene expression. When the expression ratio of a given specimen for PD-L2 was >38.0, it was considered to indicate positive *PD-L2* gene expression.

Immunohistochemistry. Immunohistochemical staining was done using Dako Envision system (DakoCytomation, Kyoto, Japan) in available 31 frozen tissues. Monoclonal antibody against human PD-L1 (MIH1, mouse immunoglobulin G1) and PD-L2 (MIH18, mouse immunoglobulin G1) was generated as previously described (13). After neutralization of endogenous peroxidase, cryostat sections on glass slides were preincubated with blocking serum and then were incubated overnight with MIH1 or MIH18. After three washes in PBS, the sections were incubated for 1 hour with biotinylated anti-mouse immunoglobulin G, washed thrice with PBS, incubated with avidin-biotinated peroxidase complex for 1 hour, and again washed for 10 minutes with PBS. Reaction products were visualized with 3,3′-diaminobenzidine tetrahydrochloride and the slides were counter-stained with hematoxylin.

Evaluation of immunostaining. All of the immunostained sections were examined under low power (4× objective) to identify regions containing low-staining tumor cells. In cases of multiple areas with low intensity, five randomly selected areas were scored, and in sections where all of the staining appeared intense, one field was selected at random. The proportion of tumor cells showing high and low staining in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per ×400 field. We set several cutoff points arbitrarily to select the best value preliminarily. When the percentage of PD-L–positive tumor cells within the tumor tissue specimen was ≧10%, significant differences were found with respect to the survival rate. Therefore, we selected 10% as the most appropriate cutoff value. Briefly, specimens with ≧10% PD-L–positive tumor cells were classified as positive. Tumor samples were examined and classified by two pathologists in a blind manner.

Statistical analysis. The overall cancer-specific survival time was calculated from the date of surgery to the date of death from esophageal cancer. The significance of the difference between PD-L expression and several clinical and pathologic variables was assessed by the χ^2 test or the Mann-Whitney *U* test. The Kaplan-Meier method was used to estimate the probability of survival, and significance was assessed by the log-rank test. Multivariate analysis was done using the Cox regression model to study six factors (PD-L status, tumor status, nodal status, metastatic status, gender, and age at surgery). In some analyses, the Spearman's rank test was also used to examine the correlation between two factors. We use the term of tumor status as T factor, nodal status as N factor, and metastatic status as M factor in tumor-node-metastasis classification, respectively. A *P* value of <0.05 was considered as statistically significant.

Results

PD-L1 and PD-L2 expression in human esophageal cancer. The quantification of *PD-L* gene expression by using real-time quantitative PCR showed that 18 (43.9%) of the 41 tumors evaluated in this study were positive for *PD-L1* or *PD-L2* gene expression and 23 (56.1%) were negative. The protein levels of PD-L1 and PD-L2 expression were also examined. The protein level of both PD-L1 and PD-L2 expression was mainly detected in the plasma membrane and cytoplasm of cancer cells (Fig. 1). A significant positive correlation between mRNA and protein expression was observed in both PD-L1 ($P = 0.019$) and PD-L2 ($P = 0.002$) from the results of 31 cases (Table 1). Therefore, we employed the quantified data of real-time PCR in the following analyses.

Correlation between PD-L expression and postoperative prognosis. We examined the relationship between PD-L expression and various prognostic factors. There was no significant relationship between either PD-L1 or PD-L2 expression and the age at surgery, gender, tumor (T) status, nodal (N) status,

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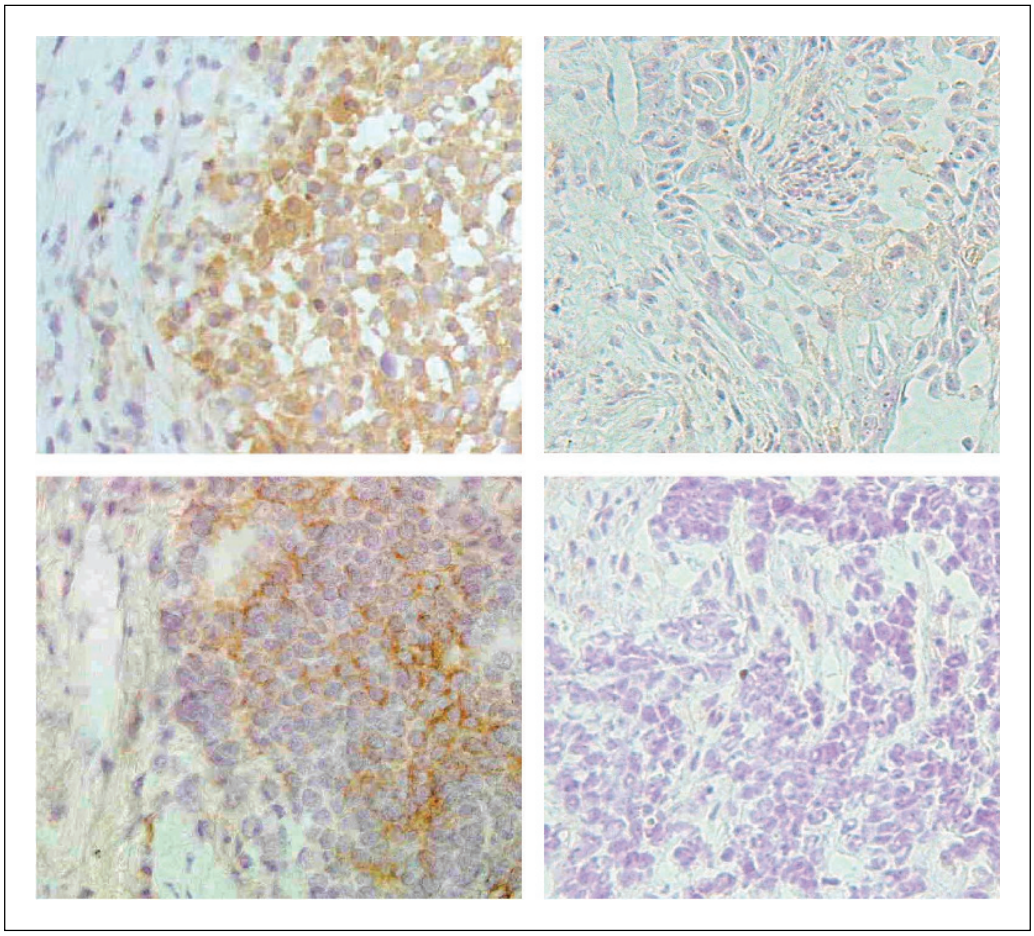

Fig. 1. Immunohistochemical staining for human esophageal cancer tissue with MIH1 recognizing PD-L1 (*top*) and MIH18 recognizing PD-L2 (*bottom*). Representative case of PD-L1 – positive (*top left*), PD-L1 – negative (*top right*), PD-L2 – positive (*bottom left*), and PD-L2 – negative (*bottom right*) tumors. Original magnification, ×200.

metastatic (M) status, or pathologic stage. Interestingly, the overall survival of PD-L1 – or PD-L2 – positive patients was significantly worse than that of negative patients ($P = 0.025$ and $P = 0.003$, respectively; Fig. 2A and B). Furthermore, 10 patients had tumors positive for both PD-L1 and PD-L2, 16 patients had tumors positive for either PD-L1 or PD-L2, and 15 patients had tumors negative for both PD-L1 and PD-L2. Overall survival of patients with tumors positive for both PD-L1 and PD-L2 was significantly worse than that with tumors negative for both (50% versus 100%, 1-year survival, $P = 0.0008$; Fig. 2C). In addition, overall survival of patients positive for either PD-L1 or PD-L2 had a tendency to be better than that with both positive and worse than that with both negative, although the differences were not statistically significant (Fig. 2C). Furthermore, we also confirmed the prognostic value of PD-L expression at protein level. Among the 31 patients evaluable for protein expression, 13 patients with tumors positive for PD-L1 protein expression had significantly poorer prognosis than 18 patients with tumors negative for PD-L1 ($P = 0.025$). In addition, 15 patients with tumors positive for PD-L2 protein expression had a poorer prognosis than 16 patients positive for PD-L2 ($P = 0.045$).

Table 1. Relationship between immunohistochemical and PCR results of PD-L1 or PD-L2 expression

	Real-time PCR PD-L1*			Real-time PCR PD-L2†		
Immunohistochemistry	Positive	Negative	Total	Positive	Negative	Total
Positive	10	3	13	12	3	15
Negative	5	13	18	3	13	16
Total	15	16	31	15	16	31

*$P = 0.019$.
†$P = 0.002$ (χ^2 test).

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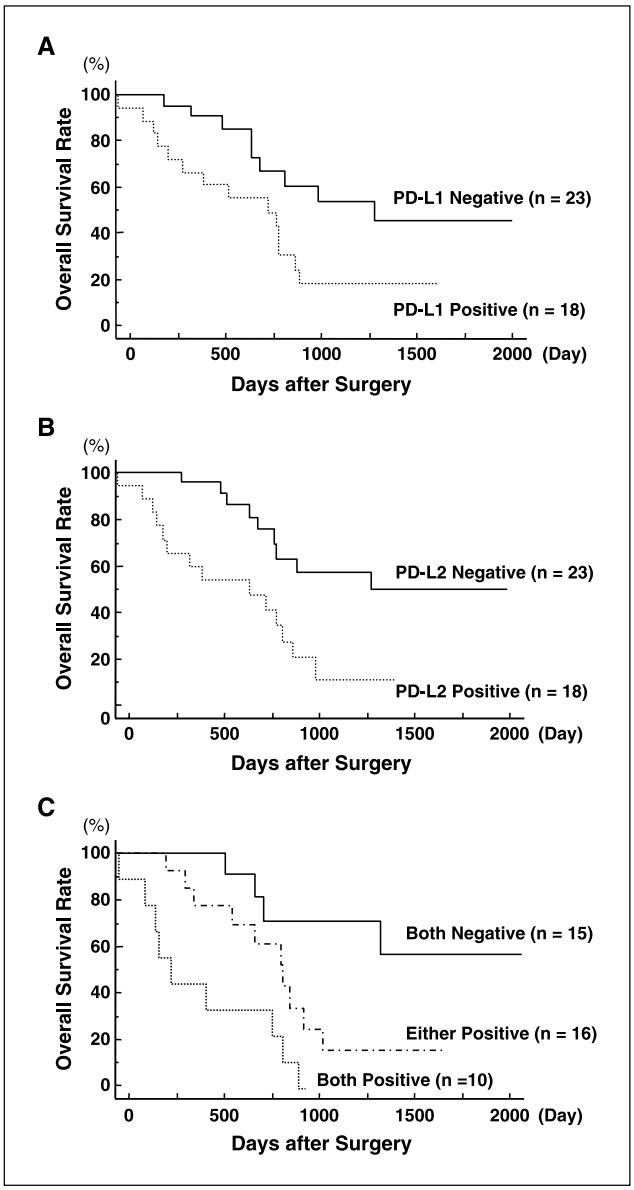

Fig. 2. *A,* overall survival of 41 patients with esophageal cancer in relation to tumor PD-L1 status. PD-L1 – positive patients had a poorer prognosis than the negative patients ($P = 0.025$). *B,* overall survival of 41 patients with esophageal cancer in relation to tumor PD-L2 status. PD-L2 – positive patients had a poorer prognosis than the negative patients ($P = 0.003$). *C,* overall survival of 41 patients with esophageal cancer in relation to tumor PD-L1 and PD-L2 status. Both PD-L1 – and PD-L2 – negative patients had a significantly better prognosis than both positive patients ($P = 0.0008$). The P value was determined by the log-rank test.

In subgroup analysis, significant differences were noted in 1-year survival rate after surgery between positive and negative patients of PD-L1 when categorized by the following variables: T_2 status, N_1 status, M_1 status, and pathologic stage of IV (Table 2). In addition, significant differences were also noted in 1-year survival rate after surgery between positive and negative patients of PD-L2 when categorized by the following variables: older patients, male patients, N_0 status, N_1 status, M_0 status, M_1 status, and pathologic stage of IV (Table 2). Furthermore, PD-L1 – and PD-L2 – positive patients with T_2, T_3 status, and stage of III showed a lower 1-year survival rate than the negative patients (Table 2). Taken together, the effect of PD-L status on the patients' postoperative prognosis was more distinct in the advanced stage of tumor compared with the early stage in clinical esophageal cancer.

Prognostic value of PD-L expression. To determine prognostic value of PD-L1 and PD-L2 expression, we did multivariate analysis using Cox regression model. PD-L1 and PD-L2 status was defined to be a significant independent prognostic factor ($P = 0.0001$). Although N status had also a significant prognostic value ($P = 0.001$), the other factors did not reach statistical significance.

Correlation between PD-L and tumor-infiltrating T lymphocytes. Furthermore, we examined the correlation between PD-L expression and tumor-infiltrating T lymphocytes (TIL). There was no significant correlation between PD-L1 expression and TILs. On the other hand, we observed statistically significant inverse correlation between PD-L2 expression and $CD8^+$ T cells ($r = -0.400$, $P = 0.011$; Fig. 3). However, no relationship was found between PD-L2 expression and CD4+ T cells (Fig. 3).

Discussion

Malignant tumors possess mechanisms for evading host immune responses. The process of evasion (so called tumor escape) may be a result of several mechanisms: (*a*) lack of T-cell recognition of tumor through impaired antigen presentation on tumor surface; (*b*) lack of T-cell recognition of tumor due to mutations in *MHC* genes or genes needed for antigen processing; and (*c*) inhibition of T-cell activation through production of immunosuppressive proteins. These properties of tumors preclude optimal immune response and permits tumor growth and metastasis *in vivo*. On the other hand, an adequate immune response against tumor may induce the activation and accumulation of immune cells and finally eliminate tumors *in vivo*. In fact, TILs are considered as a manifestation of the host immune response (21). Several clinical studies have suggested that TILs play a critical role and have prognostic significance in certain human tumors including esophageal cancer (22 – 24). Recent studies have suggested a novel mechanism that tumor may evade host immune response through the expression of PD-L1. PD-L1 and PD-L2 have been thought to be involved in the negative regulation of cellular and humoral immune responses by engaging PD-1 receptor on activated T and B cells (10, 25). In tumor immunity, tumor-associated PD-L1 has been proposed to induce apoptosis of tumor-reactive T cells (16). Thereby, tumors were thought to evade host immune response and grow *in vivo*. However, little is known about its role and importance in clinical human cancers. In this study, we investigated the clinical significance of PD-L expression in esophageal cancer, which is one of most challenging gastrointestinal tumors. Our first finding is that PD-L1 and PD-L2 were expressed in primary esophageal cancer tissues as well as in human esophageal cancer cell lines. Second, PD-L – negative patients had a significantly better prognosis than the positive patients. Third, the effect of PD-L status on prognosis was distinctive in advanced stage of cancer with positive lymph node metastasis and distant metastasis. Taken together, PD-L status may be a critical factor to promote tumor growth and metastasis in esophageal cancer. Finally,

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Table 2. One-year survival rate of 41 patients with esophageal cancer according to clinicopathologic characteristics and PD-L1 and PD-L2 status

		1-y survival rate (Å%)			1-y survival rate (Å%)		
		PD-L1			PD-L2		
	Total (*n*)	Positive	Negative	*P*	Positive	Negative	*P*
Age							
<65	24	88.9	93.3	0.321	87.5	93.8	0.204
≥65	17	44.4	100	0.064	45	100	0.007
Gender							
Male	32	64.3	100	0.079	67.1	94.7	0.003
Female	9	75	80	0.183	60	100	0.316
Tumor status							
T_1	7	66.7	100	0.702	100	83.3	0.216
T_2	20	80	100	0.008	75	100	0.103
T_3	14	40	87.5	0.249	50.8	100	0.121
Nodal status							
N_0	15	100	90.9	0.660	83.3	100	0.023
N_1	26	57.1	100	0.020	55	92.9	0.018
Metastatic status							
M_0	33	75	95.2	0.094	76.9	95	0.036
M_1	8	50	100	0.035	26.7	100	0.035
Pathologic status							
Stage I	7	100	100	0.808	100	100	0.833
Stage II	19	87.5	90.9	0.227	87.5	90.9	0.375
Stage III	7	33.3	100	0.064	50	100	0.527
Stage IV	8	50	100	0.035	26.7	100	0.035
Total	41	66.7	95.5	0.025	64.9	95.7	0.003

the most important finding in this study is that PD-L status is a significant independent prognostic factor. The underlying mechanism has not been determined yet in this study. As previously proposed, the induction of apoptosis and subsequent deletion of tumor-reactive T cells by PD-L1 may be a key mechanism (16). In this study, however, we could not find a significant correlation between PD-L1 status and TILs. Although we cannot presently exclude any possibilities, there may be some possible explanations for this discrepant data. First, PD-L1 expression in human esophageal cancer may function in tumor growth and metastasis independently of deletions of TILs. In fact, a recent report has suggested another mechanism that suppression of myeloid dendritic cell function through up-regulation of PD-L1 by tumor environmental factors may contribute to the impaired immune responses and tumor progression (26). Second, real-time PCR analysis used in this study could examine the deletion, but not the inactivation, of TILs through PD-L1 expression in tumors. In addition, it is possible that PD-L1 expression determined by real-time PCR analysis includes PD-L1–expressing cells or tissues other than cancer tissues. However, as shown in Table 1, there was significant correlation between PCR and immunohistochemical results in PD-L expression. Moreover, the PD-L expression of normal tissues or cells in immunohistochemistry was generally weak compared with PD-L–positive cancer tissues. In contrast, there was a significant inverse correlation between PD-L2 status and $CD8^+$ TILs. $CD8^+$ T cells are generally thought to play a central role in antitumor immune response and the presence of $CD8^+$ T cells has been reported as a prognostic factor in esophageal cancer (22, 23). Our present data may corroborate the proposed mechanism of tumor evasion through PD-L expression. In addition, PD-L2 may be a better target for immunotherapy of esophageal cancer rather than PD-L1 (19).

Blocking and regulating negative signal in T-cell activation may be a novel strategy for the future cancer treatment. Recently, the therapeutic efficacy of targeting CTLA-4 (CD152), which is a potent negative regulator other than PD-1 in T-cell activation and a physiologic terminator of immune responses to pathogens, self-antigens, and alloantigens, has been clinically proven (27). In that clinical study, the use of blocking CTLA-4 antibody has been reported to elicit certain antitumor effects on cancer patients without overt toxicities (27). Our data may warrant future strategy based on the manipulation of negative regulatory pathway in T-cell activation in human cancer. A recent study showing that PD-L1 blockade enhanced the therapeutic efficacy of adoptive T-cell immunotherapy for squamous cell carcinoma may further support the implication given in this study (28). Furthermore, the combination therapy of targeting PD-1/PD-L pathway with conventional antitumor reagents or newly proposed vaccination should be considered and may be more effective in clinical cancer treatment. Because the suppression of dendritic cell function through up-regulation of PD-L1 expression has been suggested to be responsible for inhibition of proper immune response in cancer patients, blocking PD-L1 may augment the effect of dendritic cell–based vaccination as a new immunotherapy

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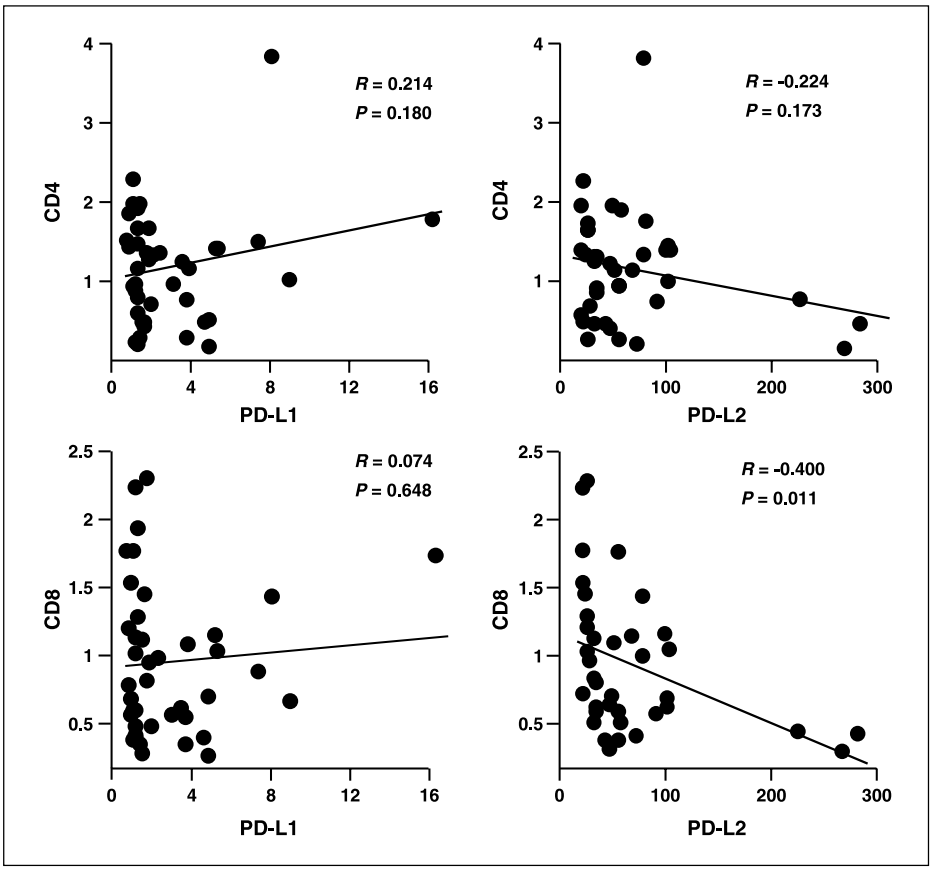

Fig. 3. Correlation between PD-L1 or PD-L2 expression and tumor-infiltrating T lymphocytes. The phenotypes of T cells were assessed as $CD4^+$ (*top*) and $CD8^+$ (*bottom*) by real-time quantitative PCR. The Spearman's rank test was used for statistical analysis.

(26). A recent study has also shown that PD-L2 cross-linking antibody can elicit considerable antitumor effect (19). These various PD-L blockade strategies may have a potential effect on postoperative patient survival.

However, there are some caveats to such a conclusion. First, recent other studies have suggested that PD-L1 could also provide positive signal through an unknown receptor other than PD-1, resulting in T-cell proliferation and induction of certain cytokines such as interleukin-10 and IFN-γ (9, 29). In addition, it has been recently shown that localized PD-L1 expression promoted organ-specific autoimmunity as well as alloimmunity (30). Furthermore, a recent study showed that PD-L2 promotes tumor immunity independently of PD-1 (18). These studies imply the considerable complexity of PD-1/PD-L pathway and the unknown receptors that interact with PD-L. Therefore, before the clinical application of targeting PD-1/PD-L pathway, additional preclinical studies and careful interpretation will be required. Second, there is fundamental difference in histologic types of esophageal cancer between Japan and the Western countries (2, 31, 32). As shown in this study, squamous cell carcinoma is the most common histologic type (over 90% of all esophageal cancer) in Japan, whereas the prevalence of adenocarcinoma has been increasing and is currently more common in the United States and Europe. Moreover, there are considerable differences in tumor malignancy and patients' prognosis between these two types of cancer. Although an adenocarcinoma cell line examined in this study also expressed PD-L, the significance of PD-L1 and PD-L2 expression in adenocarcinoma of esophageal cancer needs to be investigated.

In conclusion, we have shown for the first time that PD-L is a novel prognostic marker for human esophageal cancer. Furthermore, our data have suggested that PD-L may play a critical role in cancer metastasis and progression in humans. Because metastatic relapse is the most frequent cause of cancer-related death, our clinical data may provide the rationale of developing a novel immunotherapy targeting PD-L1 and PD-L2 for clinical treatment of esophageal cancer.

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