

PD-L1 Expression Is a Prognostic Factor in Patients with Thoracic Esophageal Cancer Treated Without Adjuvant Chemotherapy

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Abstract. *Background/Aim: Programmed death-1 ligand 1 (PD-L1) induces apoptosis of tumor-reactive T-cells, that enables tumors to evade immune defense and thus furthers their growth. Our aim was to determine whether PD-L1 expression status correlates with prognosis in patients with advanced thoracic esophageal squamous cell carcinoma. Patients and Methods: The PD-L1 expression status of 177 patients treated with esophagectomy without preoperative therapy was evaluated immunohistochemically using tissue microarray. We then statistically analyzed the relationships between PD-L1 expression status and clinicopathological features and survival. Results: In patients undergoing surgery alone, PD-L1 expression was significantly positivity associated with a better prognosis. By contrast, there were no significant correlations between PD-L1 expression and clinicopathological features or outcomes in patients treated with surgery plus postoperative adjuvant chemotherapy. Conclusion: PD-L1 positivity in advanced thoracic esophageal squamous cell carcinoma may be predictive of a positive prognosis in patients treated without adjuvant chemotherapy.*

Esophageal cancer is one of the most difficult types of gastrointestinal cancers to treat and is the sixth leading cause of death worldwide (1). Although advances in the comprehensive treatment of esophageal cancer have enabled improvement of outcomes (2-4), the extremely aggressive behavior of this cancer type continues to limit the 5-year survival rate among these patients (5).

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Programmed death-1 (PD-1) is a co-stimulatory molecule expressed on T-cells, B-cells and myeloid cells, which provides an inhibitory signal during T-cell activation (6-8). The PD-1 ligands PD-L1 and PD-L2 are cell-surface glycoproteins belonging to the B7 family (9-12). Previous studies have shown that PD-1/PD-L1 ligation inhibits T-cell growth and cytokine secretion (10, 12). Moreover, recent studies suggest that tumor-associated PD-L1 induces apoptosis in tumor-reactive T-cells, thereby enabling tumors to evade host immune defenses and grow (13). Aberrant PD-L1 expression has been detected in various human malignancies and, in most of these cases, PD-L1 expression correlates with a poor prognosis and high malignancy (14-16). On the other hand, recent studies have also suggested that PD-L1 may provide a positive signal leading to T-cell proliferation (9, 17). Consequently, the prognostic significance of PD-L1 expression in human esophageal cancer is not entirely clear. The purpose of this study was to clarify whether PD-L1 expression status correlates with the prognosis of patients with advanced thoracic esophageal squamous cell carcinoma.

Patients and Methods

Patients. During the period from January 2001 to December 2010, a total of 459 patients with esophageal cancer underwent esophagectomy at Akita University Hospital. Among these patients, we enrolled 177 with T2-4 thoracic esophageal cancer who had undergone curative esophagectomy with no pre-operative treatment. Esophageal cancer stage and the treatment strategy were defined for each patient at a conference attended by radiologists, physicians and surgeons. The disease was classified according to the International Union against Cancer tumor-node-metastasis (TNM) Classification of Malignant Tumors (seventh edition) (18).

Because this study was conducted before the publication of the JCOG9907 study (19), all the patients were treated with esophagectomy alone or esophagectomy followed by adjuvant chemotherapy which was regarded as the standard therapy at that time in Japan (20). Whether or not to administer adjuvant

chemotherapy was determined based on the pathological status and clinical condition of the patient after the surgery. The adjuvant chemotherapy was administered according to the guidelines and algorithms for esophageal cancer treatment edited by the Japan Esophageal Society (20).

Surgery. Our standard operative procedure is right transthoracic or thoracoscopic esophagectomy with two- or three-field lymph node dissection. For the patients in this study, three-field lymph node dissection of the mediastinal (involving the periesophageal region and areas around the trachea and bilateral main bronchus), abdominal (involving the perigastric region and areas around the celiac axis), and cervical (involving the bilateral periesophageal region and supraclavicular region) lymph nodes were performed. We commonly perform reconstruction by inserting a gastric tube *via* the posterior mediastinal route.

Adjuvant chemotherapy. Based on the pathological results, initial administration of adjuvant chemotherapy was started within 2 months after esophagectomy. Some patients declined adjuvant chemotherapy or were not eligible because of their clinical condition. Details of the adjuvant chemotherapy are described elsewhere (19). In brief, the chemotherapy consisted of protracted infusion of 5-fluorouracil (800 mg/m²/day) on days 1-5, combined with cisplatin (80 mg/m²/day) on day 1. This protocol was repeated twice with 3-week intervals in between. All patients were followed-up using procedures designed to detect recurrence of their cancer. Follow-up consisted of physical examination, blood tests, chest X-rays and neck/chest/abdominal computed tomography. As a general rule, patients visited the hospital every 2 months for 5 years after their surgery. Neck/chest/abdominal computed tomography was carried out every 6 months for 2 years and at least every year thereafter.

Esophageal squamous cell carcinoma tissue microarray. An esophageal squamous cell carcinoma tissue microarray (TMA) was constructed at the Pathology Institute, Toyama, Japan using 177 paraffin blocks of primary tumor. Areas of squamous cell carcinoma were identified by pathologists from hematoxylin/eosin-stained sections from each paraffin block. To account for cancer heterogeneity, three randomly selected cores measuring 0.6 mm in diameter were collected from each paraffin block and placed in the TMA.

Immunohistochemistry. Four-micrometer-thick sections from the TMA were deparaffinized in xylene and ethanol, placed in 10 mmol/l Tris buffer (pH 9.0) containing 1 mmol/l EDTA and irradiated with microwaves (750 W) for 5 min. Endogenous peroxidase activity was blocked by incubating the sections for 15 min in 3% H₂O₂, and nonspecific binding was blocked by incubation for 30 min in 10% goat serum (Nichirei, Tokyo, Japan). The specimens were then incubated for 60 min with rabbit monoclonal antibody to PD-L1 (1:200 dilution, 13684; Cell Signaling Technology, Danvers, MA, USA) as the primary antibody. This was followed by incubation first in blocking buffer and then with a peroxidase-conjugated, anti-rabbit antibody (Histofine Mouse stain Kit®; Nichirei) for 30 min each. Thereafter, the tissue sections were developed by incubation for 5 min with 3, 3'-diaminobenzidine tetrahydrochloride (Nichirei), and the antigen was visualized using biotin, horseradish peroxidase-conjugated streptavidin and 3, 3'-diaminobenzidine peroxidase substrate according to the manufacturer's instructions. Finally, the sections

were counterstained with Gill hematoxylin, dehydrated and mounted. Photomicrographs of the immunostaining were taken for analysis using a NanoZoomer Digital Pathology Virtual Slide Viewer (Hamamatsu Photonics, version 1.2.33, Hamamatsu, Shizuoka, Japan). For each tumor, the percentage of PD-L1-positive area (decimal scale from 0-100%) within the whole cancer area in three cores was determined by two surgeons blinded to the clinical data. We preliminarily arbitrarily set several cut-off points in order to select the best value. Significant differences for survival were found when the PD-L1 positive area was 10% or more within the whole cancer area. Hence samples were deemed positive when the stained area was 10% or more of the whole cancer area and patients were partitioned by this value into PD-L1-positive and PD-L1-negative groups.

Biostatistical analysis. The median and frequency were used to summarize the characteristics of the patients in the PD-L1-positive and PD-L1-negative groups. The Wilcoxon test (for continuous variables) or χ^2 and Fisher exact tests (for categorical variables) were used to compare the differences between these groups. Survival length was determined from the date of surgery to death or the date of the last clinical attendance. Survival curves were derived using the Kaplan-Meier method, and differences between curves were analyzed using the log-rank test. Cox's proportional hazards regression model was used for multivariate analyses. Tumor location, T status, N status, pathological stage, and PD-L1 expression were included in the multivariate model. All analyses were performed using JMP 10 (SAS Institute, Cary, NC, USA), which yielded two-sided *p*-values. Values of *p*<0.05 were considered significant.

Results

PD-L1 Expression in cancer cells. PD-L1 localized primarily at the cell membrane, although it was also distributed in the cytoplasm. Figure 1 shows representative images of samples staining positively (Figure 1a) and negative (Figure 1b) for PD-L1. Among the 177 patients studied, 49 (27.7%) were defined as being positive for PD-L1 expression and 128 as negative based on immunohistochemical analysis.

Clinicopathological associations of PD-L1 expression. Among the 177 patients studied, 105 received adjuvant chemotherapy after esophagectomy (surgery-adjuvant group) and 72 did not (surgery-alone group). The clinicopathological features of the patients partitioned with respect to PD-L1 expression are summarized separately in Table I. The median patient age at esophagectomy was 71 years (range=38-82 years) in the surgery-alone group and 64 (range=38-78 years) in the surgery-adjuvant group. Among those treated with surgery alone, 15 were defined as PD-L1-positive based on immunohistochemical analysis; the remaining 57 were PD-L1-negative. Statistical analysis indicated that early tumor stage and lower recurrence rate correlated with PD-L1 positivity (*p*=0.029 and *p*=0.009, respectively, Table I). Among those treated with surgery plus adjuvant chemotherapy, 34 were defined as PD-L1-positive, while the

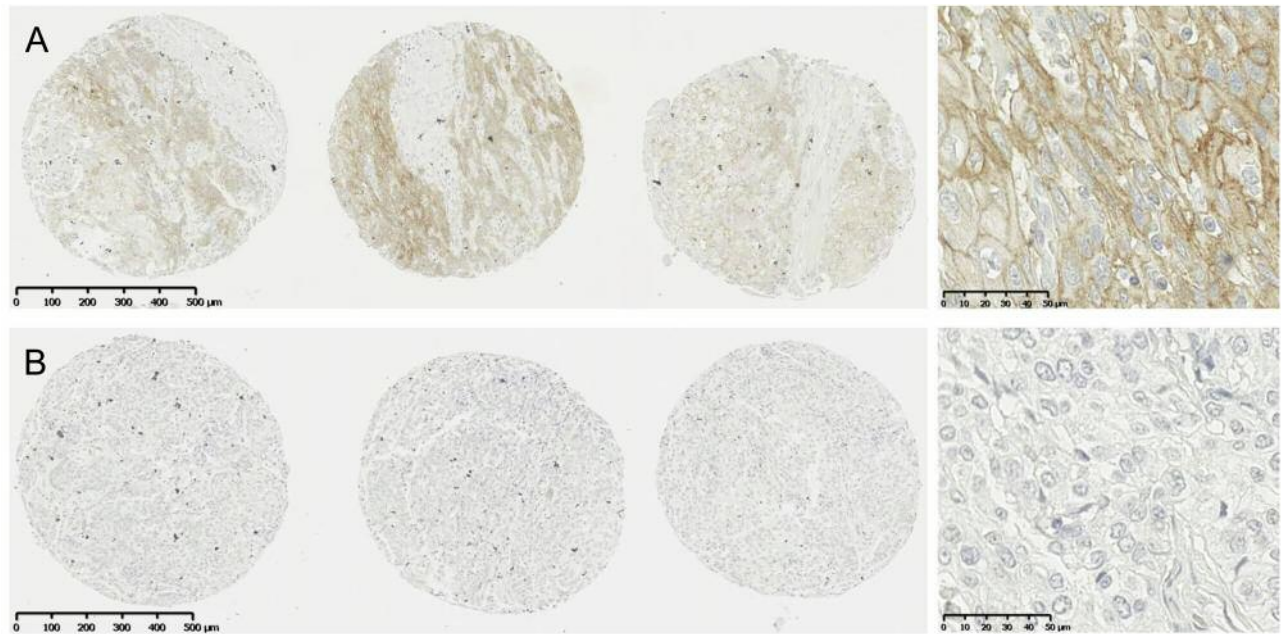


Figure 1. Photomicrographs showing exemplary immunochemical staining of triple cores from a tissue microarray revealing programmed death-ligand 1 (PD-L1) expression in esophageal squamous cell carcinoma. A: Positive expression of PD-L1. PD-L1 was localized at the membrane and in the cytoplasm. B: Negative expression of PD-L1.

remaining 71 were PD-L1-negative. There were no significant differences in the clinicopathological features between the PD-L1-positive and PD-L1-negative patients in the surgery-adjuvant group.

Prognostic effect of PD-L1 expression. In the surgery-alone group, Kaplan–Meier curves showed that 5-year disease-specific survival (DSS) and disease-free survival (DFS) were significantly better among PD-L1-positive patients ($p=0.0158$ and $p=0.0148$, respectively, Figure 2). Although the same tendency was found for 5-year overall survival (OS), the difference in OS between PD-L1-positive and -negative patients was not statistically significant ($p=0.0808$). In the surgery-adjuvant group, there were no significant differences in 5-year OS, DSS or DFS between PD-L1-positive and -negative patients. Consistent with these findings, univariate analysis of age (≥ 70 vs. < 70 years), gender, depth of invasion (T2 vs. T3-4), lymph node metastasis (N1-3 vs. N0), pathological stage (IIIA-IIIC vs. IB-IIIB), tumor differentiation (poor vs. not poor), postoperative pneumonia (yes vs. no) and PD-L1 expression (negative vs. positive) showed lymph node metastasis, pathological stage, tumor differentiation and PD-L1 expression status to be significant prognostic factors affecting 5-year DSS in the surgery-alone group ($p=0.0005$, $p=0.0005$, $p=0.0297$ and $p=0.0045$, respectively, Table II). Furthermore, in a multivariate analysis using a Cox

regression model, PD-L1 expression status was determined to be a significant independent prognostic factor ($p=0.0144$, Table III).

Discussion

In this study, we demonstrated that among patients treated with surgery without adjuvant chemotherapy for thoracic esophageal cancer, PD-L1-positive patients were found to have a significantly better prognosis than PD-L1-negative patients. On the other hand, there were no significant differences in survival among patients receiving postoperative adjuvant chemotherapy. We also showed that PD-L1 expression status was an independent prognostic factor in patients treated with esophagectomy without adjuvant chemotherapy.

The PD-L1–PD-1 interaction serves as an important regulatory check against excessive adoptive immune responses to antigens and autoimmunity (21). Thus, upon T-cell receptor activation, PD-L1 acts as a negative regulator of the immune response (22, 23). In addition, experiments using murine models showed that PD-L1 is a key mediator enabling tumor cells to evade the immune system both *in vitro* and *in vivo* (10-13, 22-24). Immunochemical treatments targeting the PD-L1–PD-1 axis are currently under investigation and have shown evidence of antitumor activity (25-27). In addition, it has been suggested that PD-L1 expression in tumor cells

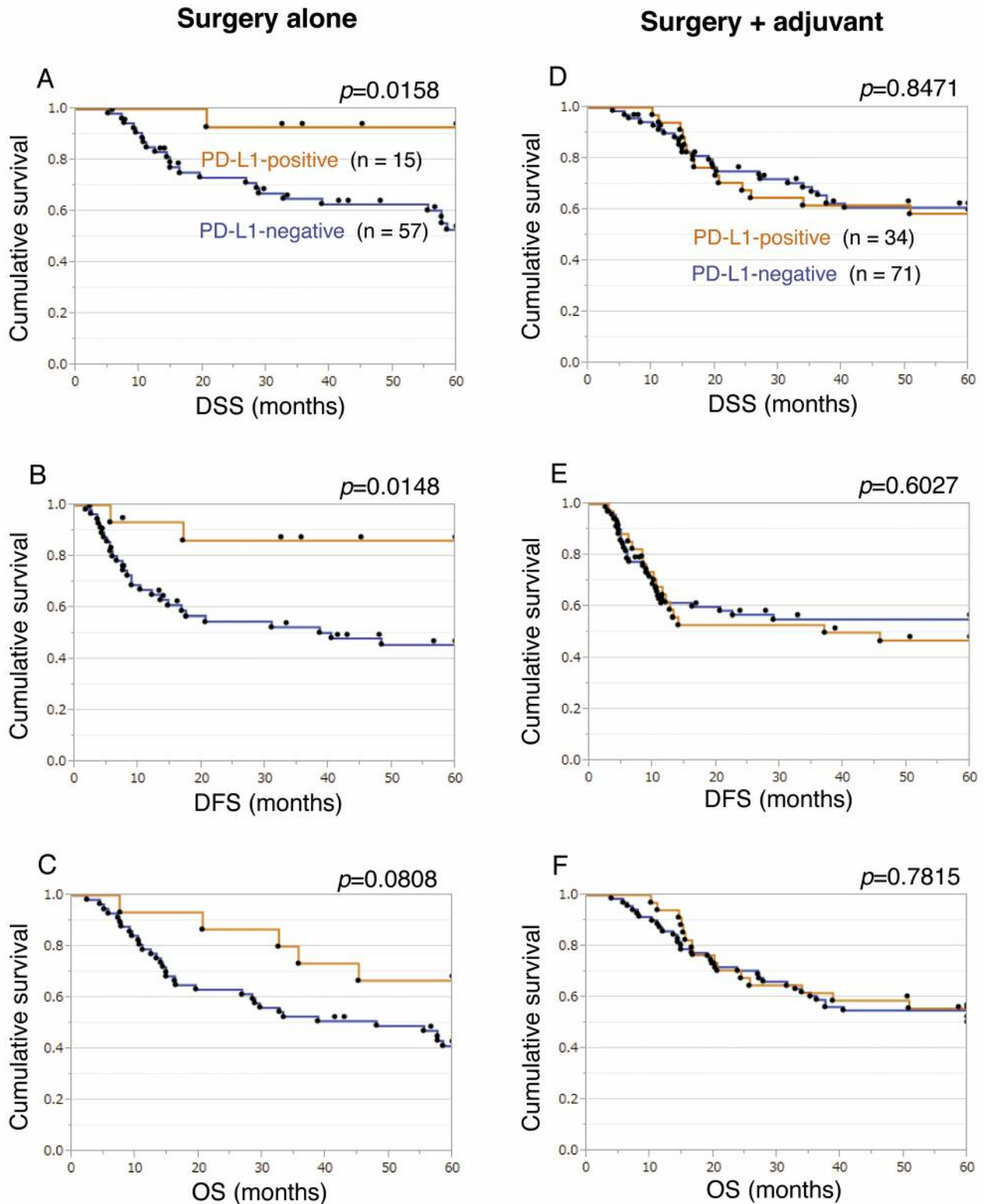


Figure 2. Kaplan-Meier curves assessing the impact of programmed death-1 ligand 1 (PD-L1) expression on disease-specific (DSS) (A, D), disease-free (DFS) (B, E) and overall (OS) (C, F) survival among PD-L1-positive and PD-L1-negative patients in the group treated with surgery alone and that treated with surgery plus adjuvant therapy. PD-L1-positive patients had significantly longer DSS (A) and DFS (B) than PD-L1-negative patients in the group treated with surgery alone. Dots above the line indicate censored cases; dots on the line indicate failed cases.

Table I. Clinicopathological features of patients with esophageal cancer (EC).

Factor	Surgery alone (n=72) PD-L1, n			Adjuvant group (n=105) PD-L1, n		
	Positive (n=15)	Negative (n=57)	p-Value	Positive (n=34)	Negative (n=71)	p-Value
Gender			0.631			0.257
Female	2	5		3	14	
Male	13	52		31	57	
Median age at surgery (range), years	70 (57-82)	71 (38-78)		62.5 (49-72)	64 (38-78)	
Tumor location			0.055			0.781
Upper	1	0		1	4	
Middle	7	40		22	47	
Lower	7	17		11	20	
Depth of invasion			0.174			0.804
T2	5	8		7	11	
T3	9	47		25	56	
T4a	1	2		2	4	
Lymph node metastasis			0.525			0.343
N0	8	20		9	12	
N1	2	4		1	5	
N2	3	16		10	17	
N3	1	6		4	19	
N4	1	11		10	18	
Pathological stage			0.029*			0.056
IB	5	2		3	0	
IIA	3	17		6	11	
IIB	0	1		1	5	
IIIA	4	19		7	27	
IIIB	1	9		6	14	
IIIC	2	9		11	14	
Tumor differentiation			0.403			0.132
Well	2	8		3	16	
Moderate	11	32		15	33	
Poor	2	17		16	22	
Adverse events			0.610			0.669
Positive	9	30		11	26	
Negative	6	27		23	45	
Anastomotic leakage			0.532			0.609
Positive	4	11		4	11	
Negative	11	46		30	60	
Recurrent nerve paralysis			0.582			0.666
Bilateral	1	1		0	1	
Unilateral	3	11		9	15	
Negative	11	45		25	55	
Pneumonia			0.372			0.059
Occurred	6	16		1	11	
Absent	9	41		33	60	
Recurrence of EC.			0.009*			0.535
Positive	2	28		18	33	
Negative	13	29		16	38	
Prognosis			0.062			0.701
Alive	9	17		16	33	
Died from EC	1	23		15	28	
Died from other cancer.	1	2		1	1	
Died from other disease	4	15		2	9	

*Statistically significant.

could serve as a predictive tool (15). However, the clinical relevance of PD-L1 expression was poorly investigated until recently (13, 24). Ohigashi *et al.* were the first to declare that

PD-L1 expression was related to a poorer prognosis in patients with esophageal cancer (28). Chen *et al.* then reported that OS was significantly poorer among patients with

Table II. Univariate analysis of 5-year disease-specific survival in patients with esophageal cancer not receiving adjuvant chemotherapy.

Variable	Univariate Cox proportional hazards model		
	HR	95% CI	p-Value
PD-L1 expression: Negative (n=57) vs. positive (n=15)	7.969	1.678-142.568	0.0045*
Age: ≥70 (n=44) vs. <70 (n=28)	1.567	0.688-3.874	0.2906
Gender: Male (n=65) vs. female (n=7)	2.656	0.560-47.517	0.2638
Depth of invasion: T2 (n=13) vs. T3-4 (n=59)	1.133	0.376-2.820	0.8059
Lymph node metastasis: Positive (n=44) vs. negative (n=28)	5.203	1.960-17.934	0.0005*
Pathological stage: IIIA-IIIC (n=44) vs. IB-IIB (n=28)	5.203	1.960-17.934	0.0005*
Tumor differentiation: Poor (n=19) vs. not poor (n=53)	2.660	1.108-6.040	0.0297*
Postoperative pneumonia: Yes (n=22) vs. no (n=50)	1.460	0.591-3.322	0.3941

PD-L1: Programmed death-1 ligand 1; HR: hazard ratio; CI: confidence interval. *Statistically significant.

Table III. Multivariate analysis of 5-year disease-specific survival in patients with esophageal cancer not receiving adjuvant chemotherapy.

	HR	95% CI	p-Value
PD-L1 expression: Negative (n=57) vs. positive (n=15)	6.677	1.361-120.601	0.0144*
Age: ≥70 (n=44) vs. <70 (n=28)	1.135	0.426-3.149	0.8012
Gender: Male (n=65) vs. female (n=7)	2.275	0.453-41.374	0.3730
Lymph node metastasis: Positive (n=44) vs. negative (n=28)	2.081	0.275-18.948	0.5153
Pathological stage: IIIA-IIIC (n=44) vs. IB-IIB (n=28)	2.362	0.317-20.691	0.4390
Tumor differentiation: Poor (n=19) vs. not poor (n=53)	1.987	0.758-5.151	0.1593

PD-L1: Programmed death-1 ligand 1; HR: hazard ratio; CI: confidence interval. *Statistically significant.

PD-L1-positive tumors than among those with PD-L1-negative ones (29). Tumor-infiltrating T-lymphocytes (TILs) are thought to be a manifestation of the host immune response to the tumor, and induction of apoptosis and subsequent elimination of TILs by PD-L1 is thought to be a mechanism by which the immune response to the tumor is suppressed. However, previous studies reported no significant correlation between PD-L1 expression and TILs, although the two cohorts were small (28, 29).

The prognostic value of PD-L1 in malignant disease is unclear, as earlier studies have reported conflicting results. Whereas a positive correlation between PD-L1 expression and prognosis was reported in non-small cell lung cancer and mismatch repair (MMR)-proficient colorectal cancer (30, 31), no relation was found between PD-L1 expression and survival in osteosarcoma, melanoma or MMR-deficient colorectal cancer (31-33), and there was correlation with adverse outcomes in gastric cancer and renal cell carcinoma (34-37).

We observed that in patients with esophageal cancer treated surgically without adjuvant chemotherapy, PD-L1 positivity is related to better 5-year DSS and DFS rates than in patients without PD-L1 expression. Similar associations were recently reported by Chen *et al.* (38), who assessed 536 patients with esophageal squamous cell carcinoma and found

that PD-L1 expression was a positive prognostic factor. They also showed that PD-L1 positivity correlated with an upper esophageal location, well-differentiated tumor, absence of lymph node metastasis and early tumor stages, which suggests PD-L1 expression is an indicator of less aggressive tumors. These results may be somewhat surprising, as expression of an immunosuppressive molecule appears to correlate with improved outcomes. Future investigations of the relation between PD-L1 and TIL function may reveal the underlying mechanism. It has been suggested that the presence of particular TIL subsets, such as CD8-positive cytotoxic T-cells, correlates with a better prognosis in various malignancies (39-46). Tumor infiltration by CD8-positive T-cells is itself an independent prognostic factor in both squamous cell carcinoma and adenocarcinoma (42). Moreover, cooperation between CD4- and CD8-positive T-cells reportedly leads to an improved prognosis in patients with esophageal squamous cell carcinoma (47). Evidence also suggests that PD-L1 may provide a positive signal *via* an as yet unknown receptor, which induces T-cell proliferation and secretion of interleukin-10 and interferon- γ , thereby activating antitumor effects (9, 17). In addition, localized PD-L1 expression reportedly promotes organ-specific autoimmunity as well as alloimmunity (48).

An important finding in this study is that contrary to the results in patients undergoing surgery alone, there was no significant difference in survival between PD-L1-positive and -negative patients treated with surgery plus adjuvant therapy. This suggests chemotherapy not only has a direct cytotoxic effect on tumor cells, it also affects the tumor immune system. We conjecture that tumoral expression of PD-L1 may be reduced by chemotherapy in patients with esophageal cancer. Although the relationship between tumoral PD-L1 expression and chemotherapy has not been fully investigated, Lesterhuis *et al.* reported that platinum dephosphorylates signal transducer and activator of transcription 6, resulting in decreased PD-L2 expression in both human dendritic cells and tumor cells (49).

The clinical significance of PD-L1 expression in esophageal cancer has not yet been firmly established. Therefore, clarification of the distribution and expression rate of PD-L1 in cancer and its clinical relevance are important issues for future investigation.

Conclusion

PD-L1 expression was detected in 27.7% of patients with esophageal squamous cell carcinoma. In contrast to earlier findings that PD-L1 expression contributes to suppression of antitumor immune responses, our study indicates that PD-L1 expression in squamous cell esophageal carcinoma has a positive impact on the prognosis of patients treated by esophagectomy without adjuvant chemotherapy.

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