Original Article

Clinical relevance of PD-L1 and PD-L2 overexpression in patients with esophageal squamous cell carcinoma

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Background: Even with the advance of diagnosis and the treatment, the 5-year survival rate for esophageal cancer patients is still poor. The checkpoint protein inhibition provides another choice to improve the survival. The expression of the programmed death ligand-1 (PD-L1) was reported but the clinical relevance remained inconsistent in esophageal cancer. Besides, there were few references about the other ligand, programed death ligand-2 (PD-L2). In this study, we evaluated the expressions of PD-L1 and PD-L2 in patients with esophageal squamous cell carcinoma (ESCC) and assessed their clinical relevance.

Methods: From 1996 to 2011, 150 patients undergone complete surgical resection for ESCC were enrolled. Clinical data were recorded. Expression of PD-L1 and PD-L2 on cytoplasm in paraffin embedded tumor samples were analyzed by immunohistochemistry staining and scored with a semi-quantitative method.

Results: Of the patients, 96 (64.0%) patients had PD-L1 overexpression and 63 (42.0%) had PD-L2 overexpression. There was a correlation between the expression of PD-L1 and PD-L2 ($P<0.001$). Patients without overexpression of PD-L1, pathological T1–2 and N0 status, pathological stage I–II and no post-operative adjuvant treatment had a better disease free survival (DFS). In multivariate analysis, PD-L1 expression and pathological stage were the independent prognostic factors for DFS. The expression of PD-L2 did not influence the DFS. Although not statistically significant, patients without overexpression of PD-L1 and PD-L2 seem to have a better overall survival (OS).

Conclusions: The overexpression of PD-L1 on cytoplasm, not PD-L2, is an independent prognostic factor for DFS in patients with ESCC undergone esophagectomy. However, there is a trend which suggested that patients without overexpression of PD-L1 and PD-L2 had a better OS.

Keywords: Programmed death ligand-1 (PD-L1); programed death ligand-2 (PD-L2); overexpression; cytoplasm; esophageal squamous cell carcinoma (ESCC); survival

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Introduction

According to the report of the World Health Organization, the incidence of esophageal cancer is increasing and it is the 6th most common cancer in males and the 13th common cancer in females (1). The main treatment methods for esophageal cancer include surgical resection, chemotherapy, and radiotherapy. However, even the treatment methods of esophageal cancer have advanced greatly in recent decades, the results are still poor, and the 5-year survival rate is less than 15% (1-4). Over the last 10 years, the development of immunotherapy provides another choice to treat the disease and improve the survival.

Programmed death-1 (PD-1) is a co-inhibitory receptor expressed on the cell surface of T cells, B cells, monocytes and natural killer cells. PD-1 is part of the CD28 signaling family with structure similar to cytotoxic T lymphocyte antigen-4 (CTLA-4), especial in extracellular domain (5,6). Programmed death ligand-1 (PD-L1, B7-H1) and programmed death-1 ligand-2 (PD-L2, B7-DC) are the ligands for PD-1; both have been identified as glycoprotein on the cell surface which belongs to the B7 receptor family (7-10). Although these two ligands have partial identity and similarity in structure, their regulation for expression is different (9,11).

Some recent studies show that the interaction between PD-1 and programmed death ligand (PD-L) inhibits T-cell receptor signaling and down-regulates T-cell response (8,10,12). Other studies further suggested an inhibitory role for the PD-1 and PD-L engagement in the responses of lymphocyte and regulation of peripheral tolerance using PD-1–deficient mice model (6,13). In addition to these basic immunologic roles, recent studies have suggested that PD-L1 in cancer cell increases apoptosis of T cells *in vitro* and the PD-L1 expression promotes tumor growth *in vivo* (14). Preclinical study suggested PD-L1 is upregulated by tumor cells and by cells in the tumor microenvironment (15). Furthermore, blockade of the PD-1 and PD-L1 interaction can restore T cell activity against tumor cells to prevent cancer metastasis and reduce tumor volume (12,13,16). These previous studies are well evaluated the function of PD-L1, but the clinical implication is still unknown (14,16). In the other part, the expression of PD-L1 is reported in various different human cancers, such as lung, liver, colon and also in melanomas, however, the association between PD-L1, patient's clinicopathological characteristics and prognosis has not been determined, especially in esophageal cancer (14,17-20).

On the other hand for PD-L2, although PD-L2 expression has been reported in esophageal adenocarcinoma, the function in tumor cells remains unclear. There were several publications have suggested that PD-L2 expression may play a role in tumor immunity (21-23). However, the clinical relevance was still unknown.

Therefore, the aim of this study was to investigate the expression of PD-L1 and PD-L2 in patients with esophageal squamous cell carcinoma (ESCC) post resection to define the clinical significance.

Methods

From January 1996 to December 2011, patients with ESCC that underwent operation for tumor resection with reconstruction at Taipei Veterans General Hospital were included. The patients who had the history of preoperative chemoradiotherapy or death within 30 days after surgery were excluded. This study was approved by the Institutional Review Board of Taipei Veterans General Hospital. Patient informed consent was waived due to the retrospective nature of the study. Research was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

Clinical data collection

The medical records of patients were retrospectively reviewed. The clinical data of patients were collected from the medical records, such as age, sex, smoking status, pathological factors and the results of follow-up studies. Pathological TNM staging was determined according to the 7th ed. UICC/AJCC TNM staging system (24). Follow-up studies, including computerized tomography scans of the chest and the brain, and whole body nuclear scan studies, were performed every 3–6 months within 5 years and then annually. Disease-free survival (DFS) was calculated from the date of surgery to the time of the first relapse (recurrence or metastasis). Overall survival (OS) was defined as the period from the date of surgery to the date of death due to any cause.

Immunohistochemical staining procedures and interpretation

The paraffin blocked specimens were gained from Tissue Bank of Taipei Veterans General Hospital. Slides with 4 μm thick were used for immunohistochemical staining to examine the expression of PD-L1 and PD-L2. Briefly, tissue sections were treated initially in a decloaking chamber with

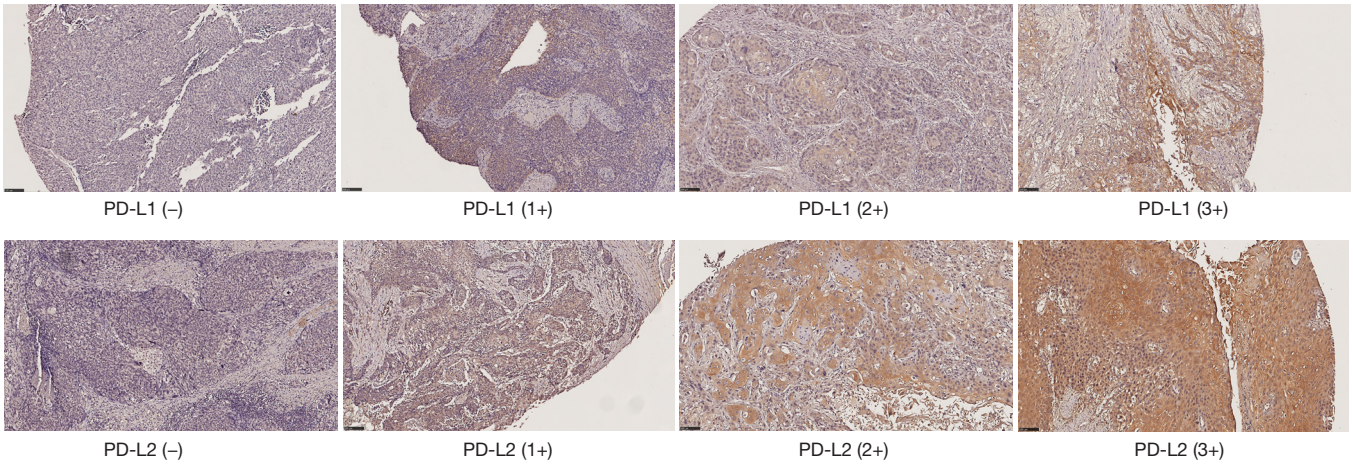

Figure 1 Representative images of PD-L1 and PD-L2 expression in esophageal cancer tissues by immunohistochemical staining. The expression of PD-L1 (upper row) and PD-L2 (lower row) was scored from 0 to 3+ (left to right). Magnification: ×100.

a sodium citrate buffer (10 mM, pH 6.0) and then with serum blocking solution (Histostain Bulk Kit; Invitrogen, Carlsbad, CA) to remove endogenous peroxidase activity and to reduce nonspecific background staining. Mouse anti-human monoclonal antibody to PD-L1 (1:50, 329702, BioLegend) and PD-L2 (1:50, 329602, BioLegend) were then applied to the tissue sections. Slides were incubated at 4 ℃ overnight in a moist chamber. The tissue sections were subsequently treated with biotinylated secondary antibody for 30 min, DAB substrate solution (K4065, DakoCytomation) for 1–3 min, and then counterstained with hematoxylin for 5 min.

The expression of PD-L1 and PD-L2 on the cytoplasm of tumor cells was evaluated by a pathologist and semi-quantified by the predominant staining intensity of the tumor cells. The staining grading was scored a 0 (no staining), 1+, 2+, and 3+, shown in *Figure 1*. Comparing with 0 or 1+ staining, patients with 2+ and 3+ staining were grouped as the overexpression.

Statistical analysis of immunohistochemical staining and clinicopathological data

Chi-square tests were used to compare the categorical factors. Numerical values were expressed as mean ± SD and were compared using the Student's *t*-test. The survival curves were plotted by Kaplan-Meier method and compared using the log-rank test. The impacts of clinicopathological factors and expression of PD-L1/PD-L2 on survival were assessed using Cox proportioned hazards regression method. A $P<0.05$ is defined as statistical significance. All statistical analyses were performed using SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL).

Results

A total of 160 patients who had undergone esophagectomy with reconstruction for ESCC were enrolled in this study. Six patients received pre-operative concurrent chemoradiotherapy and 4 patients died within 1 month after operation were excluded. There were 150 patients included in final analysis. The mean age of the 150 patients was 64.1±10.8 years, ranging from 36 to 88 years. Of these patients, 137 (91.3%) were male and 13 were female. Half of the patients had a history of smoking (75, 50%) with a smoking index ranging from less than 5 to 80 pack-years. Seventy (46.7%) patients had their tumor located at middle portion of their thoracic esophagus, followed by 60 patients with a lower portion tumor. A cervical or upper thoracic esophagus tumor was found in 20 patients.

All patients received an esophagectomy with reconstruction, 112 (74.7%) through open thoracotomy, 16 (10.6%) through thoracoabdominal incision, 15 with thoracoscopic assistance, which included two with robotic assistance, and 7 patients had a transhiatal approach to complete the tumor resection. Except one patient using colon for reconstruction, the other 149 patients used gastric tube for reconstruction. The route used in 105 (70.0%) patients was the retrosternal route and 45 involved the posterior mediastinal route. All patients had a cervical

anastomosis except one patient had an intrathoracic anastomosis.

Based on the AJCC classification for esophageal cancer, 9 (6.0%) patients had pathological T1 lesions, 25 (16.7%) T2, 103 (68.7%) T3, and 13 (8.7%) T4 lesions. The average number of lymph nodes removed was 24.5 (ranging 2-87). For pathological N status, 53 (35.3%) patients had no nodal involvement, 50 (33.3%) N1, 27 (18.0%) N2, and 20 (13.3%) N3 status. For pathological stages, 14 (9.3%) patients had stage I, 44 (29.3%) stage II, 87 (58.0%) stage III, and 5 (3.3%) stage IV disease. The pathological stages I–II correlated with the pathological T1–2 status (P<0.001) and N0 status (P<0.001). In this series, the range of tumor length was 1.0–14 cm with median 4.4 cm. Tumor length was significantly shorter in the pathological early T status (P=0.001) and stage I–II (P=0.013). Due to local advance or lymph nodes involvement, 61 (40.7%) patients received adjuvant treatment postoperatively. There were 35 patients received concurrent chemoradiotherapy, 11 chemotherapy and 15 radiotherapy alone. Patients with long tumor length (P=0.001), pathological T3–4 (P=0.006), nodal involvement (P<0.001) and late stage disease (P<0.001) had a higher opportunity to receive adjuvant treatment.

According to the assessment of the immunohistochemical staining, 96 (64.0%) patients had PD-L1 overexpression and 63 (42.0%) had PD-L2 overexpression. There was a correlation between the expression of PD-L1 and PD-L2 (P<0.001). The expression of PD-L1 was also correlated with patients without post-operative adjuvant therapy (P=0.037). There were no other significant correlations between clinicopathological parameters and the expression of PD-L1 and PD-L2, as shown in *Table 1*.

The median follow-up time was 23.3 months [interquartile range (IQR) 10.7–67.6 months]. Prior to data analysis, 92 (61.3%) patients had tumor relapse. The 3- and 5-year DFS rates were 45.4% and 39.8%, respectively. The univariate and multivariate analyses of DFS are presented in *Table 2*. Patients without overexpression of PD-L1 (P=0.013, *Figure 2*), pathological T1–2 and N0 status, pathological stage I–II and no post-operative adjuvant treatment had a better DFS. The expression of PD-L2 did not influence the DFS (P=0.101, *Figure 3*). In multivariate analysis, PD-L1 expression and pathological stage were the independent prognostic factors for DFS.

A total of 86 (57.3%) patients those died of the esophageal cancer disease within five years. Another 38 patients died of other disease, most reasons were poor nutrition and pulmonary problems. The 3- and 5-year OS rates were 36.7% and 27.9%, respectively. The univariate and multivariate analyses of OS are presented in *Table 3*. Patients with a well differentiation tumor, pathological nodal negative status and pathological early stage had a better OS. Patients without overexpression of PD-L1 and PD-L2 had a better OS, but not significant (*Figures 2,3*). Pathological stage was the independent prognostic factor for OS in multivariate analysis.

Discussion

Immunotherapy has been heralded as a major breakthrough in cancer treatment in recent years. As the basic principle of modern immunotherapy, immune check-point blockage using PD-1 and PD-Ls antibodies appears to be one of the most popular immunotherapy approaches (25).

PD-L1 and PD-L2 are the two known ligands for PD-1. PD-L1 expression has been reported in many different human neoplasms, such as lung, breast, gastric, pancreatic, kidney, bladder, ovarian cancers, and melanoma (26). PD-L1 expression has been correlated with rapid cancer progression, higher recurrence rates and poorer survival rates. Recently, there are several publications that have shown the relationship between PD-L1 expression and esophageal cancer, including both ESCC and esophageal adenocarcinoma, but the results were inconsistent (27). Investigations focused on PD-L2 in malignant tumors are still rare. Thus, most of the studies have focused on the expression of PD-L1 with inconsistent results and the influence of the expression of PD-L2 in tumor tissues remains unknown.

In this study, 64.0% of patients had PD-L1 overexpression and 42.0% had PD-L2 overexpression. There was a significant correlation between the expression of PD-L1 and PD-L2 (P<0.001), but there were complete no correlations between the clinicopathological parameters and the expression of PD-L1 and PD-L2. Only postoperative adjuvant therapy status was negatively correlated with PD-L1 expression. These findings are similar to some previous studies, shown in *Table 4*. However, it has been reported in different studies that overexpression of PD-L1 is correlated with age, tumor site, tumor differentiation, tumor length, tumor depth, pathological T, N, stage status, and history of pre-operative treatment or response, but without full consistence (28-43).

The overexpression rates of PD-L1 in various studies are different, between 14.5% in the study of Zhang and his colleagues in Tianjin, China and 82.8% in the study of

Table 1 Characteristic correlations between PD-L1 (over *vs.* non-over) and PD-L2 (over *vs.* non-over) expression

Variable	PD-L1			PD-L2		
	Non-over (n=54)	Over (n=96)	P	Non-over (n=87)	Over (n=63)	P
Age	63.8	64.2	0.804	64.0	64.2	0.925
Gender			0.847			0.365
Male	49	88		81	56	
Female	5	8		6	7	
Smoking			0.736			0.870
No	26	49		44	31	
Yes	28	47		43	32	
Tumor length (cm)			0.903			0.547
<4	27	47		44	30	
>4	27	49		43	33	
Differentiation			0.616			0.083
Well	5	9		10	4	
Moderate	31	54		52	33	
Poorly	10	8		9	9	
No mention	8	25		16	17	
pT status			0.759			0.371
T1–2	13	21		22	12	
T3–4	41	75		65	51	
pN status			0.463			0.262
N0	17	36		34	19	
N1–3	37	60		53	44	
Pathological stage			0.462			0.069
I–II	23	35		39	19	
III–IV	31	61		48	44	
Post-op adjuvant treatment			0.037			0.588
No	26	63		50	39	
Yes	28	33		37	24	

PD-L1, programmed death-1 ligand-1; PD-L2, programmed death ligand-2.

Chen *et al.* in Jiangsu, China (29,39). The overexpression rate of PD-L1 in our study was 64.0%, just between their reports. There were five studies that included patients with pre-operative chemo/radiotherapy, while our study had only chemo/radiotherapy-naive patients. Interestingly, PD-L1 expression negatively influenced the survival in these five studies, including OS in 4 studies and distal failure rate only in the other study (30,34,35,37,41).

The overexpression rate of PD-L2 (42.0%) in our study is little lower than other three studies (42.5% to 48.4%), but not much different. There was no survival impact of PD-L2 expression on both disease free survival (DFS) and OS. The result was the same as those of Leng and his coworkers in OS (36). In contrast, two studies from Ohigashi *et al.* and

Table 2 Univariate and multivariate analysis of factors influencing disease free survival (DFS) after surgical resection

Variable	Univariate		Multivariate	
	5-year rate (%)	P value	HR (95% CI)	P value
PD-L1 expression		0.013		0.042
Non-over	57.7		1 (referent)	
Over	28.7		1.713 (1.020–2.880)	
PD-L2 expression		0.101		0.277
Non-over	44.6		1 (referent)	
Over	32.7		1.319 (0.801–2.173)	
Age (years)		0.275		
<65	34.1			
>65	47.0			
Gender		0.334		
Male	39.3			
Female	48.0			
Smoking		0.091		0.097
No	49.4		1 (referent)	
Yes	30.1		1.485 (0.931–2.369)	
Tumor length (cm)		0.166		
<4	45.0			
>4	34.1			
Differentiation		0.147		
Well	41.0			
Moderate	40.1			
Poorly	23.7			
No mention	48.5			
pT status		0.045		0.988
T1–2	55.9		1 (referent)	
T3–4	34.2		0.994 (0.437–2.260)	
pN status		0.002		0.531
N0	56.7		1 (referent)	
N1–3	29.2		1.351 (0.527–3.461)	
Pathological stage		<0.001		<0.001
I–II	60.1		1 (referent)	
III–IV	22.5		2.507 (1.499–4.194)	
Post-op adjuvant treatment		0.039		0.279
No	46.7		1 (referent)	
Yes	30.5		1.340 (0.789–2.274)	

HR, hazard ratio; CI, confidence interval; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2.

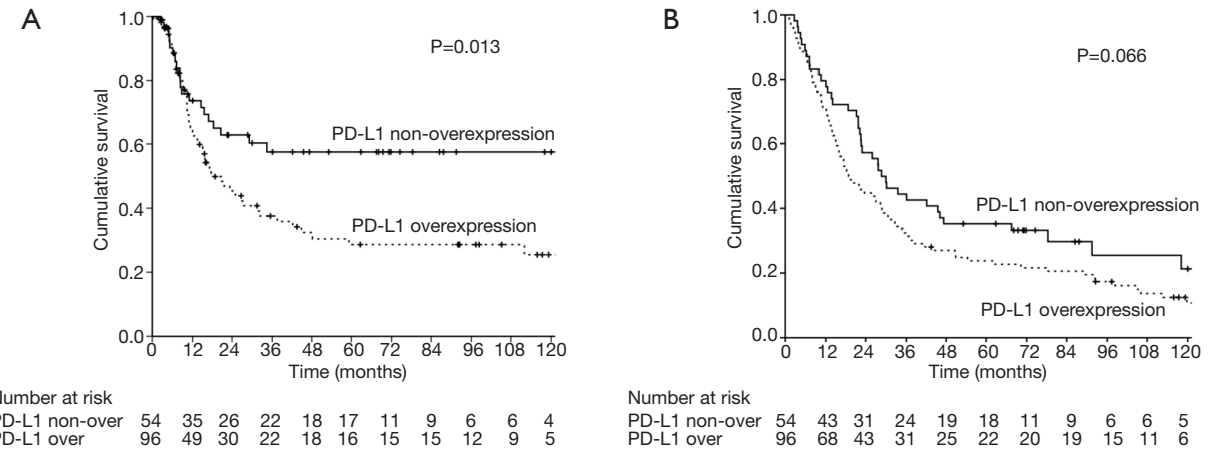

Figure 2 The survival curves in patients with esophageal squamous cell carcinoma, solid line: PD-L1 non-overexpression; dotted line: PD-L1 overexpression. (A) Disease free survival; (B) overall survival

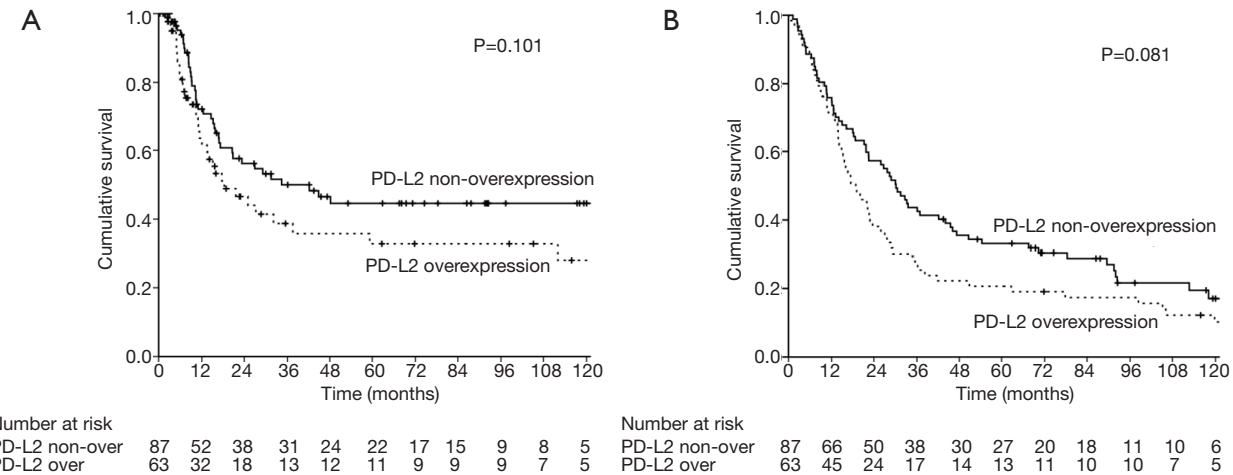

Figure 3 The survival curves in patients with esophageal squamous cell carcinoma, solid line: PD-L2 non-overexpression; dotted line: PD-L2 overexpression. (A) Disease free survival; (B) overall survival.

Tanaka *et al.* showed that expression of PD-L2 negatively influenced the OS in univariate analysis (28,34).

In the analysis of DFS, overexpression of PD-L1 is an independent prognostic factor for worse survival using multivariate analysis in this study. There were ten studies previously that investigated the influence of PD-L1 in DFS in patients with ESCC. Five studies had the same results as ours, even though only univariate analysis was used in two studies (35,37,38,40,42). However, two studies had the opposite result; in these studies the patients with positive expression of PD-L1 had a better DFS rate (31,43). The other studies showed the expression of PD-L1 had no influence on DFS.

In the OS analysis, there was not significantly different between patients with and without overexpression of PD-L1 using the Kaplan-Meier method in this study (P=0.066). In the past, there were three of 16 studies had the same results as our study (31,37,39). Most studies showed that expression of PD-L1 was negatively influencing the OS with four studies using multivariate analysis. Only two studies in Japan and Germany disclosed that expression of PD-L1 was a better independent predictor for prognosis (33,43), shown in *Table 4*.

In general, the heterogeneity in patient populations in the different studies, such as age, gender, pathological factors and the percentage of chemo/radiotherapy-naive

Table 3 Univariate and multivariate analysis of factors influencing overall survival (OS) after surgical resection

Variable	Univariate		Multivariate	
	5-year rate (%)	P value	HR (95% CI)	P value
PD-L1 expression		0.066		0.166
Non-over	35.2		1 (referent)	
Over	23.8		1.316 (0.892–1.941)	
PD-L2 expression		0.081		0.536
Non-over	33.1		1 (referent)	
Over	20.6		1.129 (0.770–1.655)	
Age (years)		0.374		
<65	30.3			
>65	25.6			
Gender		0.644		
Male	26.9			
Female	38.5			
Smoking		0.090		0.063
No	33.3		1 (referent)	
Yes	22.3		1.425 (0.981–2.070)	
Tumor length (cm)		0.310		
<4	32.2			
>4	23.7			
Differentiation		0.042		0.084
Well	42.9		1 (referent)	
Moderate	31.6		1.233 (0.655–2.320)	0.516
Poorly	16.7		1.634 (0.763–3.500)	0.206
No mention	18.2		2.010 (1.012–3.994)	0.046
pT status		0.090		0.574
T1–2	43.7		1 (referent)	
T3–4	23.2		0.853 (0.490–1.485)	
pN status		0.001		0.839
N0	47.0		1 (referent)	
N1–3	17.5		0.922 (0.421–2.018)	
Pathological stage		<0.001		<0.001
I–II	49.8		1 (referent)	
III–IV	14.1		2.173 (1.476–3.197)	
Post-op adjuvant treatment		0.381		
No	31.3			
Yes	23.0			

HR, hazard ratio; CI, confidence interval; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2.

Table 4 Comparisons of clinical implications and PD-L1 expression in patients with esophageal squamous cell carcinoma

First author, year	Case number	Pre-operative treatment	Staining location	PD-L1 overexpression (%)	Correlated factor	DFS impact of PD-L1 overexpression	OS impact of PD-L1 overexpression
This study	150	No	Cytoplasm	64.0	No factor	Worse[b]	ND
Ohigashi, 2005 (28)	31	Unknown	Membrane/ cytoplasm	41.9	N.A.	NA	Worse[a]
Chen, 2014 (29)	99	No	Membrane/ cytoplasm	82.8	N.A.	NA	Worse[a]
Lim, 2016 (30)	73	Yes	Membrane/ cytoplasm	56.2	No factor	ND	Worse[b]
Chen, 2016 (31)	536	No	Membrane/ cytoplasm	41.4	Tumor site, differentiation, N status, stage	Improved[a]	ND
Ito, 2016 (32)	90	No	Membrane/ cytoplasm	18.9	Tumor site, tumor depth, N status	ND	Worse[a]
Hatogai, 2016 (33)	196	No	Membrane	18.4	Age	NA	Improved[b]
Tanaka, 2016 (34)	180	Some	Cytoplasm	29.4	N status, pre-operative treatment	NA	Worse[b]
Chen, 2016 (35)	162	Yes	Membrane/ cytoplasm	45.7	Tumor depth, nodal status, pre-op treatment response	Worse[a]	Worse[b]
Leng, 2016 (36)	106	No	Membrane/ cytoplasm	46.2	No factor	NA	Worse[a]
Kim, 2016 (37)	200	Some	Membrane/ cytoplasm	33.5	No factor	Worse[a]	ND
Zhu, 2017 (38)	133	No	Membrane/ cytoplasm	51.1*	Age, tumor length, differentiation,	Worse[b]	Worse[b]
Zhang, 2017 (39)	344	No	No mention	14.5	T status, stage	ND	ND
Jiang, 2017 (40)	428	No	Membrane	79.7	N status	Worse[b]	Worse[b]
Momose, 2017 (41)	251	Some	Membrane	15.5	No factor	NA	Worse[a]
Tsutsumi, 2017 (42)	90	No	Membrane/ cytoplasm	63.3	T status	Worse[a]	Worse[a]
Jesinghaus, 2017 (43)	125	No	Membrane	30.4	No factor	Improved[b]	Improved[b]

[a], by univariate analysis; [b], by multivariate analysis; *, including tumor cells and tumor infiltrating lymphocyte. PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; DFS, disease free survival; OS, overall survival; ND, no difference; NA, not applicable.

patients may influence the results of PD-Ls, such as positive rate and the impaction of survival. But, the difference in the criteria for "Positive" staining in tissue samples across studies is the major reason with the inconsistent results. This has been pointed out by Dhupar and his Pittsburgh group (27). Several different antibodies that recognize the different part of PD-Ls would influence the reading of staining. A significant variation of cutoffs for positive staining, including intensity and tumor percentage, would also make the results confuse and would make the comparisons between studies quite difficult. There is a need for a standard scoring system to consider and compare the clinical outcomes in the future.

In addition, the staining in different cells of tissue samples, the tumor cells *vs.* the tumor infiltrating immune cells, or in different locations of tumor cells (cytoplasm and membrane) may represent different effects that may have the different meaning. Although PD-L1 is a membranous protein, the production of PD-L1 comes from the cytoplasm of cells. The membranous and/or cytoplasmic staining of PD-L1 was common presented in most of the previous studies (28-32,35-38,42). There were also focused on membranous staining only in several studies (33,40,41,43). In this study, we only focused on the expression of PD-L1 on the cytoplasm of tumor cells, not the membranous or cytoplasmic/membranous staining, that was the same as the study of Tanaka *et al.* (34), but differed with other studies.

In conclusion, the expression of PD-L1 on cytoplasm is an independent prognostic factor for DFS in patients undergoing esophagectomy for ESCC, but PD-L2 expression is not. There is a trend which suggested that patients without overexpression of PD-L1 and PD-L2 had a better OS.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by the Institutional Review Board of Taipei Veterans General Hospital. Patient informed consent was waived due to the retrospective nature of the study. Research was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments.

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