



Original contribution

PD-L1 expression on tumor cells associated with favorable prognosis in surgically resected esophageal squamous cell carcinoma ^{☆,☆☆,★}



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Summary There is limited evidence regarding the relationship between programmed cell death ligand 1 (PD-L1) expression on tumor cells (TCs) and prognosis of esophageal squamous cell carcinoma (ESCC). This retrospective study aimed to investigate the clinical significance of PD-L1 expression in ESCC. To assess PD-L1 expression, we conducted immunohistochemistry studies using a tissue microarray encompassing 233 ESCC cases, stages I, II, and III, with detailed clinical data. PD-L1 expression on TCs was observed in 55.4% (129/233) of ESCC cases and was not associated with clinicopathological factors. ESCC patients with PD-L1-positive tumors showed significantly better overall survival and disease-free survival than did those with PD-L1-negative tumors ($P = .023$ and $P = .026$, respectively). When patients were stratified into those with stage I-II (127; 54.5%) and stage III (106; 45.5%) disease and those without (134; 57.5%) and with (99; 42.5%) lymph node metastasis, the prognostic effect was inconsistent. The overall survival and disease-free survival of patients with positive PD-L1 expression were significantly better in patients with stage I-II disease ($P = .021$ and $P = .015$, respectively) and without lymph node metastasis ($P = .009$ and $P = .07$, respectively) than their counterparts. Our results showed that PD-L1 expression on TCs was an independent predictor of prognosis of ESCC patients. However, the effect varied in patients with different stages and lymph node status. Positive PD-L1 expression was a favorable predictor in ESCC patients with

Abbreviations: ESCC, esophageal squamous cell carcinoma; PD-1, programmed cell death receptor 1; PD-L1, programmed cell death ligand 1; IRS, immunoreactivity scoring system; TCs, tumor cells; OS, overall survival; DFS, disease-free survival; TMA, tissue microarray; H&E, hematoxylin and eosin.

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stage I-II disease or without lymph node metastasis but not in patients with stage III disease or lymph node metastasis.

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1. Introduction

ESCC, a histopathological subtype of esophageal cancer, is the sixth most common type of cancer and is one of the leading causes of cancer-related death in China [1,2]. Although multidisciplinary therapies have been applied to the treatment of ESCC, the 5-year survival rate is less than 20% [2,3]. Therefore, it is imperative for researchers to identify better biomarkers and therapeutic strategies for the disease.

PD-1 is a member of the B7-CD28 costimulatory factor superfamily, which is expressed on the surface of T cells, B cells, and natural killer cells [4]. PD-L1, which is expressed on both TCs and immune cells, plays an important role in blocking the “cancer immunity cycle.” [4]. Binding of PD-L1 to PD-1 can suppress T-cell migration, proliferation, and secretion of cytotoxic mediators, and restrict TC killing [5-8]. Blockade of immune checkpoints, especially via inhibition of the PD-1/PD-L1 axis, has recently emerged as a new therapeutic tool in several types of cancers [9-12], including ESCC [13,14]. However, not all patients attained a long-term and durable response to anti-PD-L1/PD-1 agents. Studies have shown that the expression of PD-L1 on TCs is positively correlated with the efficacy of anti-PD-1/PD-L1 therapy [15].

In ESCC, the clinical relevance of PD-L1 expression on TCs remains unclear. The pooled results of our previous meta-analysis, which included 13 studies with 2777 patients, illustrated that positive PD-L1 expression on TCs was associated with poor OS but not with poor DFS [16]. To verify the above results, we designed the present study. We used a TMA that included 233 well-documented, clinically annotated ESCC specimens in the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences (CAMS), to investigate the clinical significance of PD-L1 expression on TCs.

2. Materials and methods

2.1. Patients

We retrospectively reviewed a database of medical records of 233 consecutive patients who underwent curative esophagectomy with R0 resection for histologically verified ESCC between December 2005 and June 2009 at the National Cancer Center/Cancer Hospital, CAMS. Patients with preoperative chemotherapy/radiotherapy and with distant metastasis were excluded from this study. All tissue specimens used in this study were obtained from the tissue bank of our hospital, and

all patients provided informed consent before surgery. This study was approved by the institutional review board of the National Cancer Center/Cancer Hospital, CAMS.

The clinicopathological data, including age, sex, tumor location, tumor differentiation, T stage, lymph node metastasis, and TNM stage, were recorded. The pathological classification of the primary tumor and the degree of lymph node metastasis were assessed according to the American Joint Committee on Cancer staging manual (eight edition) [17]. Patients were followed up in the outpatient department every 3 to 6 months for the first 2 years after surgery and then annually. The follow-up included documentation of the patients' medical history, physical examinations, chest computed tomography, and endoscopy (if necessary). The last follow-up was on April 5, 2017.

2.2. Tissue microarrays

Formalin-fixed, paraffin-embedded ESCC tissue samples were H&E stained and reviewed by 2 pathologists independently. The TMA was constructed by tissue blocks of 233 cases of ESCC tissues from the National Cancer Center/Cancer Hospital, CAMS. In brief, all samples of a respective tumor region were extracted from areas harboring a high tumor/stroma ratio. A series of 4- μ m-thick sections were cut and transferred to adhesive slides according to the manufacturer's instructions.

2.3. Immunohistochemistry of PD-L1

Briefly, TMA sections (4 μ m) were deparaffinized, rehydrated, treated with 2 N HCl for 15 minutes, and treated with 100 mM Tris-HCl (pH 8.5) for 10 minutes. Subsequently, the sections were blocked with 3% H₂O₂ for 30 minutes and goat serum at room temperature for 30 minutes. After blocking, the sections were incubated with rabbit anti-PD-L1 monoclonal antibody (1:200, ab205921; Abcam, Cambridge, United Kingdom) at 4°C overnight and then incubated with polyclonal peroxidase-conjugated antirabbit IgG (Zhongshanjinqiao, Beijing, China) at room temperature for 20 minutes according to the manufacturer's instructions.

2.4. Evaluation of immunostaining

Two experienced pathologists who were blinded to the clinical data independently calculated the IRS by combining the intensity category and the percentage category [18]. The intensity category of immunostaining was graded as follows:

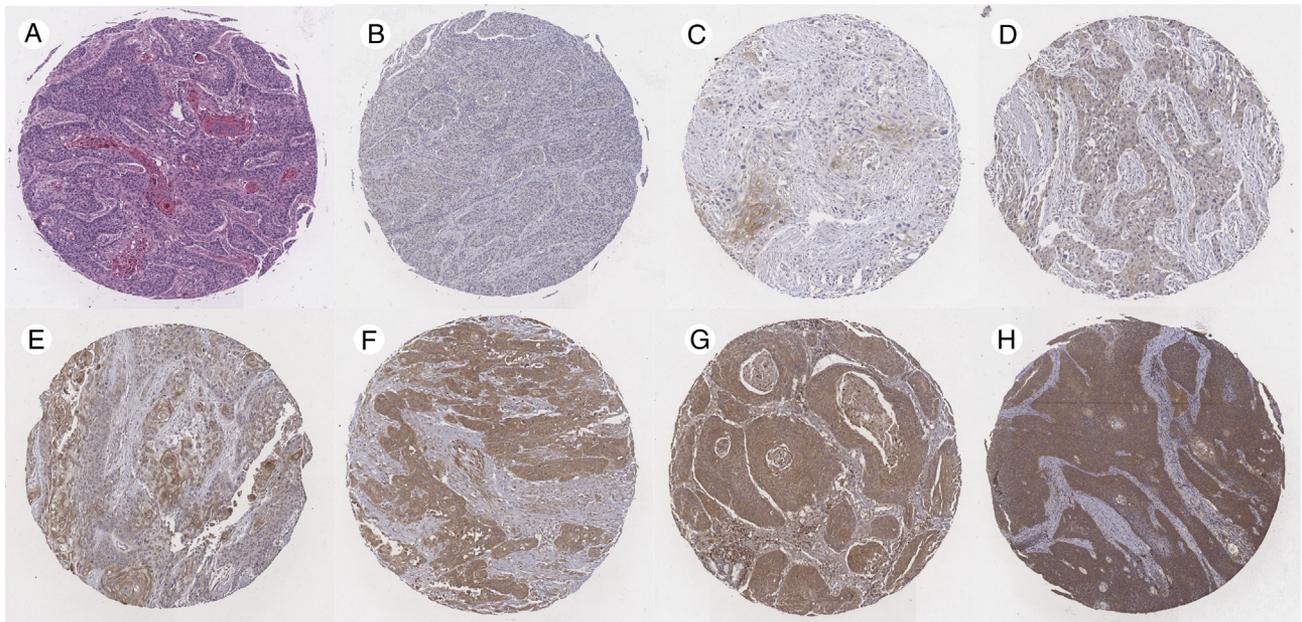


Fig. 1 Representative photomicrographs of ESCC TMA sections. A, H&E-stained TMA section. B-D, Representative photomicrographs stained with PD-L1 with IRS scores of 0, 1, and 2, respectively. E-H, Representative photomicrographs stained with PD-L1 with IRS scores of 3 or greater.

Table 1 Association of PD-L1 expression with clinicopathological factors in 233 ESCC patients

Category	Cases, no. (%)	PD-L1 expression in TCs		<i>P</i>
		Low (n = 104)	High (n = 129)	
Age (y)				.692
≤60	121 (51.9)	56	65	
>60	112 (48.1)	48	64	
Sex				.764
Male	173 (74.2)	76	97	
Female	60 (25.8)	28	32	
Smoking				.341
Ever	149 (63.9)	63	86	
Never	84 (36.1)	41	43	
Tumor location				.775
Upper	49 (21.0)	24	25	
Middle	121 (51.9)	52	69	
Lower	63 (27.1)	28	35	
Differentiation				.841
Well	64 (27.5)	28	36	
Moderate	128 (54.9)	56	72	
Poor	41 (17.6)	20	21	
T stage				.457
T1	8 (3.4)	4	4	
T2	32 (13.7)	12	20	
T3	167 (71.7)	73	94	
T4	26 (11.2)	15	11	
N stage				1.000
N0	134 (57.5)	60	74	
N+	99 (42.5)	44	55	
TNM stage				.679
I	7 (9.8)	4	3	
II	120 (42.5)	51	69	
III	106 (47.6)	49	57	

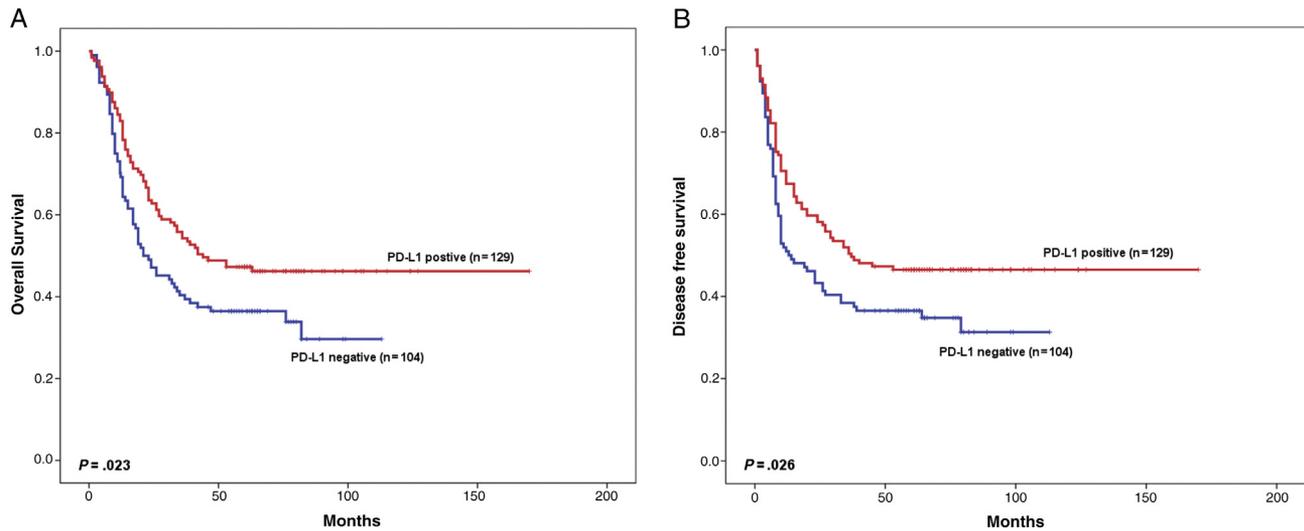


Fig. 2 Kaplan-Meier curves showing survival of patients with ESCC according to PD-L1 expression. OS (A) and DFS (B).

0 (no immunostaining), 1 (weak), 2 (moderate), or 3 (strong). The percentage category was graded as follows: 0 (negative), 1 (1%-30%), 2 (31%-60%), and 3 (61%-100%). The intensity category and the percentage category grades were multiplied, resulting in an IRS ranging from 0 to 9. In this study, the expression of PD-L1 was classified as negative (IRS, <3) or positive (IRS, ≥ 3).

2.5. Statistical analysis

Statistical analyses were performed using SPSS 19.0 (IBM, Armonk, NY). Cross-tabulations of clinical data and marker expressions were analyzed using χ^2 test or Fisher's exact test. OS and DFS were analyzed using the Kaplan-Meier method. Risk factors for the prognosis of ESCC patients were calculated by univariate Cox regression, and those with *P* values up to .1 were included in a multivariate Cox regression to identify independent prognostic variables. A *P* value less than .05 was considered statistically significant.

3. Results

3.1. PD-L1 expression on TCs in tissue samples

PD-L1 was located primarily on the cell membrane of TCs, although it was also distributed in the cytoplasm. Fig. 1 shows a representative image of H&E-stained TMA section (Fig. 1A), negatively stained TMA sections (Fig. 1B-D; IRS = 0, 1, and 2, respectively), and positively (Fig. 1E-H; IRS ≥ 3 , respectively) of PD-L1 expression on TCs. PD-L1 expression on immune cells was not evaluated. Among the 233 patients studied, 129 (55.4%) were defined as being positive for PD-L1 expression, and 104 (44.6%) were negative.

3.2. Association of PD-L1 expression with clinicopathological factors in 233 ESCC patients

The clinicopathological factors of the 233 ESCC patients grouped by PD-L1 expression are summarized in Table 1. In this cohort, the median age of the patients at

Table 2 Univariate analysis of risk factors for prognosis of 233 ESCC patients

	OS			DFS		
	<i>P</i>	HR	95% CI	<i>P</i>	HR	95% CI
Age (≤ 60 y, > 60 y)	.131	1.295	0.926-1.812	.171	1.264	0.904-1.768
Sex (female, male)	.309	1.216	0.839-1.763	.329	1.203	0.830-1.744
Smoking (ever, never)	.166	1.284	0.897-1.838	.171	1.285	0.897-1.839
Tumor location (up/middle, low)	.264	0.800	0.541-1.183	.239	0.790	0.534-1.169
Differentiation (well/moderate, poor)	.015 *	1.666	1.105-2.512	.015 *	1.664	1.103-2.509
T stage (T1/T2, T3/T4)	.014 *	1.920	1.139-3.235	.011 *	1.967	1.167-3.314
Lymph node metastasis (negative, positive)	<.001 *	2.793	1.982-3.936	<.001 *	2.806	1.994-3.950
TNM stage (I/II, III)	<.001 *	2.949	2.084-4.173	<.001 *	3.008	2.127-4.254
PD-L1 (negative, positive)	.025 *	0.681	0.487-0.953	.029 *	0.689	0.492-0.963

Abbreviations: CI, confidence interval; HR, hazard ratio.

* *P* < .05 is significant.

Table 3 Multivariate analysis of risk factors for prognosis of 233 ESCC patients

	OS			DFS		
	<i>P</i>	HR	95% CI	<i>P</i>	HR	95% CI
Differentiation (well/moderate, poor)	.050 *	1.509	0.999-2.280	.036 *	1.554	1.029-2.348
T stage (T1/T2, T3/T4)	.144	1.490	0.873-2.544	.119	1.530	0.897-2.609
Lymph node metastasis (negative, positive)	.366	1.447	0.650-3.220	.443	1.371	0.612-3.071
TNM stage (I/II, III)	<.001 *	2.862	2.021-4.054	<.001 *	2.920	2.063-4.133
PD-L1 (negative, positive)	.035 *	0.697	0.498-0.976	.045 *	0.710	0.507-0.993

Abbreviations: CI, confidence interval; HR, hazard ratio.

* *P* < .05 is significant.

diagnosis was 60 years, ranging from 36 to 85 years. One hundred seventy-three patients (74.2%) were male, and 66 patients (25.8%) were female. The median follow-up time was 34 months (1-170 months), and 98 patients (42.1%) died during follow-up. There were no significant differences between clinicopathological features and PD-L1 expression (all *P* > .05).

3.3. Survival analysis

The OS and DFS of ESCC patients according to PD-L1 expression are shown in Fig. 2. The OS and DFS were significantly higher in patients with positive PD-L1 expression than in patients with negative PD-L1 expression (*P* = .023 and *P* = .026, respectively; Fig. 2A and B).

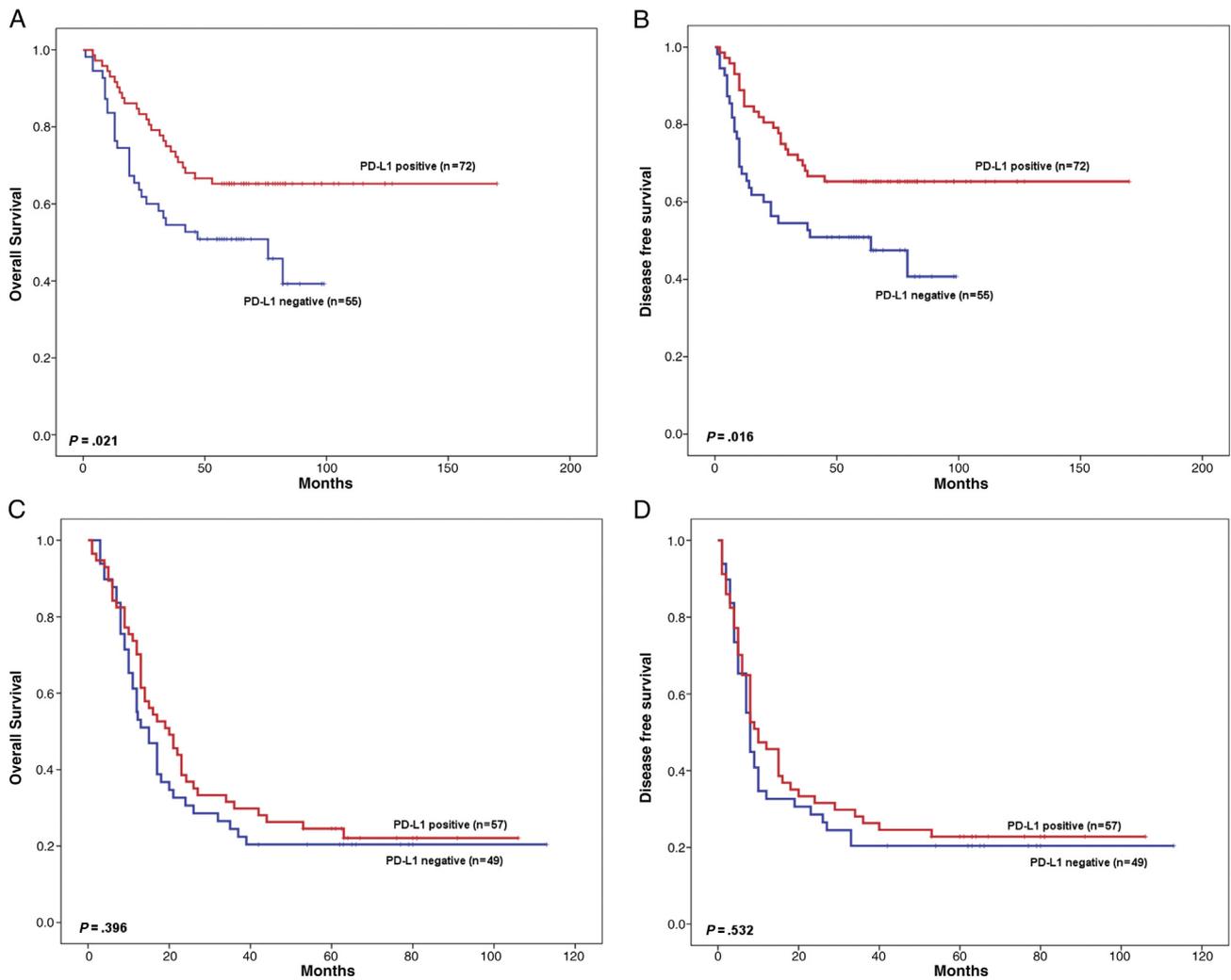


Fig. 3 Survival analyses based on clinical stage of ESCC. In patients with stage I-II disease, the PD-L1 expression was associated with improved OS (*P* = .021; A) and DFS (*P* = .016; B). However, in patients with stage III disease, the PD-L1 expression was not associated with OS (*P* = .396; C) and DFS (*P* = .532; D).

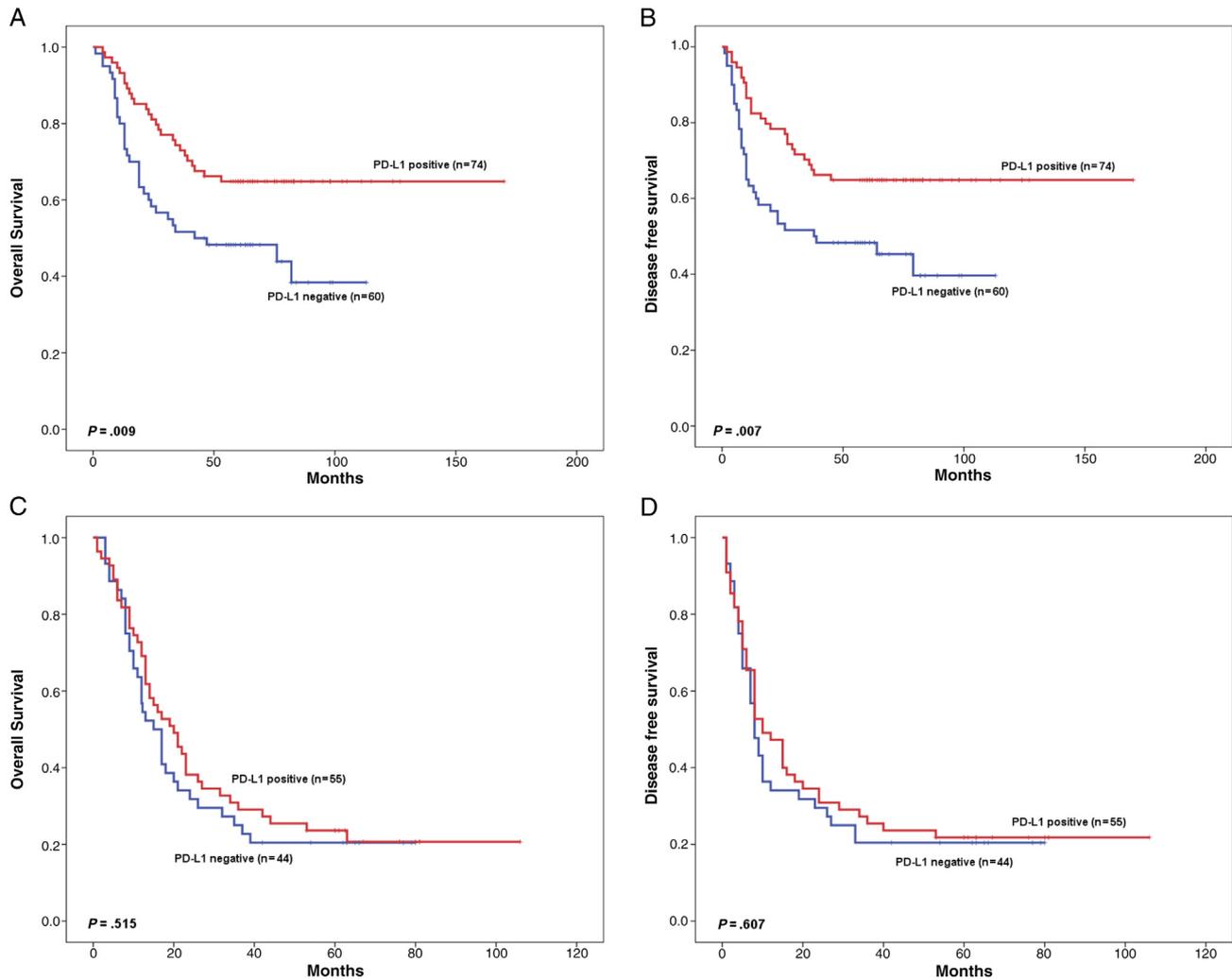


Fig. 4 Survival analyses based on lymph node status of ESCC. In patients without lymph node metastasis, the PD-L1 expression was associated with improved OS ($P = .009$; A) and DFS ($P = .007$; B). However, in patients with lymph node metastasis, the PD-L1 expression was not associated with OS ($P = .515$; C) and DFS ($P = .607$; D).

We used the univariate analysis to investigate the risk factors for both OS and DFS. The results showed that tumor differentiation, T stage, lymph node metastasis, TNM stage, and PD-L1 expression were associated with both the OS and DFS of ESCC patients (all $P < .05$; Table 2). Then, the multivariate analysis was used to investigate the independent risk factors. Only tumor differentiation, TNM stage and PD-L1 expression were independent prognostic factors of both OS and DFS (all $P < .05$; Table 3).

Moreover, we divided the 233 patients into different groups according to their TNM stage or lymph node status. In patients with stage I-II ESCC, PD-L1 expression was associated with better OS ($P = .021$) and DFS ($P = .016$; Fig. 3A and B). However, in patients with stage III ESCC, PD-L1 expression on TCs was not associated with OS ($P = .396$) or DFS ($P = .532$; Fig. 3C and D). In patients without lymph node metastasis, PD-L1 expression was associated with improved OS ($P = .009$) and DFS ($P = .007$; Fig. 4A and B). However, in patients with lymph node metastasis, PD-L1 expression was

not associated with better OS ($P = .515$) or better DFS ($P = .607$; Fig. 4C and D).

4. Discussion

There is limited evidence regarding the relationship between PD-L1 expression and prognosis in ESCC. To examine the possible role of PD-L1 expression on TCs in ESCC patients, TMAs that included 233 well-documented, clinically annotated ESCC specimens in the National Cancer Center/Cancer Hospital, CAMS, were examined by immunohistochemistry staining. We found that positive PD-L1 expression on TCs was independently correlated with significantly better OS and DFS among ESCC patients.

Almost all previous studies have used immunohistochemistry. PD-L1 expression was most commonly evaluated by the percentage of TCs with positive staining [19-24]. However, because the percentage category does not reflect the intensity

of PD-L1 expression, the IRS (the intensity category grade multiplied by the percentage category grade) was used in this study; and IRS of 3 or greater was considered to be PD-L1 positive. As a result, the rate of positive PD-L1 expression was 55.4% in the present study.

The associations between PD-L1 expression and clinicopathological factors were inconsistent with previous studies. Chen et al [25] reported that PD-L1 expression was associated with tumor stage and the stage of lymph node metastasis. However, several studies have shown that no clinicopathological features were associated with PD-L1 expression [18,20-22,26,27]. In our study, we did not find any association between PD-L1 expression and clinicopathological factors.

As a prognostic predictor for ESCC, the impacts of PD-L1 expression still remained unclear, with some studies showing better survival [20-22,27] and others showing worse survival [24,28-30]. In our previous meta-analysis, we included 13 studies with 2777 patients and found that positive PD-L1 expression was associated with poor OS [16]. However, in the present study, we found that positive PD-L1 expression was correlated with better OS and better DFS. Moreover, we divided the 233 patients into different groups according to their TNM stage or lymph node status to evaluate the prognostic value of PD-L1 expression. We found that in patients with earlier stage ESCC (stages I-II or without lymph node metastasis), PD-L1 expression was associated with a significantly better OS and DFS, whereas there was a lack of association between PD-L1 expression and clinical outcome in patients with later stage disease (stage III or with lymph node metastasis). This suggested that the prognostic value of PD-L1 expression was conditioned and not useful in all stages of ESCC. Our findings are in line with previous observations, where high PD-L1 expression on TCs was associated with better outcomes in earlier-stage ESCC [22]. The association between PD-L1 expression and a better clinical outcome has been observed in several entities other than ESCC, including colorectal carcinoma [31], non-small cell lung cancer [32], melanoma [33], and breast cancer [34]. The mechanism by which high PD-L1 expression contributes to a better clinical outcome in patients with solid cancer is still unclear. It may be reasonable to hypothesize that PD-L1 expression represents the presence of an antitumor response. In the early stages of cancer, tumor growth may still be partially suppressed and thus lead to better survival. In the later stages of cancer, however, the resistance mechanisms will ultimately become invalid and allow TCs to survive and progress. Thus, positive PD-L1 expression has an inconsistent prognostic role in ESCC patients. When addressing conflicting results in survival analyses in the future, the TNM stage or lymph node status should be taken into account.

There were some limitations in this study. First, it was a retrospective study of a population from a single institution, and thus, there is potential for selection bias. Second, although we analyzed a large cohort, the number of patients in each stage was relatively small. Therefore, our results would be best answered in the context of a multicenter, prospective study with

a large sample size. Third, this study focused exclusively on PD-L1 expression on TCs, and PD-L1 expression on immune cells was not considered. Fourth, we examined PD-L1 expression in TMA sections to evaluate its prognostic value. Biopsies capture only a small tissue volume, and tumors may heterogeneously express PD-L1, which may lead to an overestimation or underestimation of true PD-L1 levels.

5. Conclusions

In conclusion, we have demonstrated that PD-L1 expression on TCs was present in 55.3% of ESCC patients and that it was not associated with clinicopathological factors. Positive PD-L1 expression was independently correlated with significantly better OS and better DFS in ESCC patients. However, the association was inconsistent in patients with different stages and lymph node status. Positive PD-L1 expression was a favorable predictor in ESCC patients with stage I-II disease or without lymph node metastasis but not in patients with stage III disease or lymph node metastasis. This suggests that the prognostic value of PD-L1 expression should be analyzed according to different TNM stages and lymph node metastasis status.

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