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Association of the programmed death ligand-1 combined positive score in tumors and clinicopathological features in esophageal cancer

Ziling Huang[1,2] | Yan Jin[1,2] | Xu Cai[1,2] | Lijun Chen[1,2] | Xuxia Shen[1,2] | Bin Li[2,3] | Haiquan Chen[2,3] | Yuan Li[1,2]

[1]Department of Pathology, Fudan University Shanghai Cancer Center, Shanghai, China

[2]Department of Oncology, Shanghai Medical College, Fudan University, Shanghai, China

[3]Department of Thoracic Surgery, Fudan University Shanghai Cancer Center, Shanghai, China

Correspondence
Yuan Li, Department of Pathology and Oncology, Fudan University Shanghai Cancer Center, Shanghai, 200032, China.
Email: whliyuan@hotmail.com
Haiquan Chen, Department of Thoracic Surgery and Oncology, Fudan University Shanghai Cancer Center, Shanghai, 200032, China.
Email: hqchen1@yahoo.com

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Abstract

Background: The combined positive score (CPS) of the programmed death ligand-1 (PD-L1) 22C3 assay is a predictive marker of pembrolizumab monotherapy for advanced esophageal cancer (EC) patients. However, little is known about the association of the PD-L1 22C3 CPS with the clinicopathological features and heterogeneity of PD-L1 expression in EC in the Chinese population in a real-world setting.

Methods: We examined the association of the PD-L1 22C3 CPS with clinicopathological characteristics in 533 EC specimens. Further, we compared 37 cases' different blocks of the same specimen and 50 paired primary/metastatic lymph node lesions to investigate the heterogeneity of PD-L1 expression.

Results: PD-L1 positive expression was observed in 45.0% of 533 EC patients, including 46.8% with squamous cell carcinoma, 15.4% with adenocarcinoma, 28.6% with basaloid squamous carcinoma, 42.9% with spindle cell carcinoma, and 33.3% with neuroendocrine tumors. PD-L1 positive expression was positively associated with lymph node metastasis (59.2% chance, $p = 0.021$) and venous/lymphatic invasion (66.3% chance, $p = 0.029$). PD-L1 expression was highly consistent in different paraffin blocks of the same surgically resected specimen (concordance rate: 86.5%, $p = 0.000016$) and a moderate consistency (concordance rate: 78.0%, $p = 0.000373$) for the primary and metastatic lymph node lesion comparison.

Conclusions: This is a novel study which demonstrated a positive correlation between a high PD-L1 22C3 CPS and invasion/metastasis risk in EC surgical specimens. Both paired blocks and paired primary/metastatic lymph node lesions showed significant concordance. PD-L1 heterogeneity was inferred to be mainly related to positive mononuclear inflammatory cells (MICs), which might have substantial implications for clinical practice.

KEYWORDS
clinicopathological features, esophageal cancer, heterogeneity, large Chinese cohort, PD-L1 22C3 CPS

INTRODUCTION

Esophageal cancer (EC) is a major global health challenge, ranking as the sixth most common cancer worldwide,[1–3] and approximately 246 000 new cases were diagnosed in 2015 in China (177 000 males and 69 000 females).[4] Patients with advanced esophageal cancer have a poor prognosis and there are few effective therapeutic agents for EC.[5–7] EC is the fourth and sixth leading cause of mortality for males and females, respectively, in China, of which a total of 188 000

Ziling Huang, Yan Jin and Xu Cai contributed equally to this study.

persons died from EC.[4] Similarly, the 5 year relative survival rate was also extremely low (approximately 20%) from 2009 through 2015 in the United States.[8] With the development and application of immunotherapy, immune checkpoint inhibitors may be beneficial for patients with advanced EC–based on results from two large clinical trials called KEYNOTE-180 and KEYNOTE-181.[9,10] In 2019, the immune checkpoint inhibitor pembrolizumab was approved by the Food and Drug Administration (FDA) as a second-line treatment for patients suffering locally advanced or metastatic esophageal squamous cell carcinoma (ESCC) with programmed death ligand-1 (PD-L1) positively expressed in tumors (combined positive score [CPS] ≥10). More recently, KEYNOTE-590 supports profoundly improved survival when pembrolizumab combined chemotherapy is added in first-line treatment of patients, who have esophageal squamous cell carcinoma (ESCC) with combined positive score ≥ 10 tumors.[11,12] In addition, CheckMate 577 provides proof for the improvement in disease-free survival after received nivolumab adjuvant therapy in resected patients, who had esophageal or gastroesophageal junction carcinoma and received neoadjuvant chemoradiotherapy before.[12,13]

In EC, most published reports about the association between clinicopathological features and PD-L1 expression have used different PD-L1 antibodies and TPS scoring algorithm,[14–18] and the CPS of the PD-L1 22C3 assay (PD-L1 22C3 CPS) has only been evaluated in clinical trials.[9,10] To the best of our knowledge, the evaluation of the PD-L1 22C3 CPS in surgical resection specimens has not been performed. In addition, as immunotherapy selection relies on accurate CPS evaluation, a comprehensive understanding of PD-L1 expression is needed to better predict the response to immunotherapy in patients. Some studies have reported that heterogeneous PD-L1 expression may partly explain this controversy and could invalidate the use of PD-L1 expression as a predictive marker for treatment selection in other cancers.[10,19–23] However, in EC patients, pathological analysis of the heterogeneity of PD-L1 expression has not been performed. A previous study noted that the RNA level of the CD274 gene (PD-L1) was highly amplified in focal EC patient cells, which led to strong expression of PD-L1.[24,25] Similarly, one study only compared PD-L1 SP142 expression in adenocarcinoma between primary tumors and metastases by tissue-array.[26] This finding indicated that the patients might harbor variable PD-L1 expression. The heterogeneity of PD-L1 expression raises the concern that the CPS on one slide may not be representative of overall expression in the biopsy sample. Thus, sampling error may incorrectly classify the PD-L1 expression status, and heterogeneity may also have a direct impact on EC patients considering immunotherapy.

In this study, we summarized and comprehensively analyzed 533 EC cases, identifying the clinicopathological characteristics related to the PD-L1 22C3 CPS. We then further assessed the heterogeneity and concordance between two paired paraffin blocks from the same surgical resection samples and between paired primary and lymph node metastatic lesions. A model of PD-L1-positive cells was also analyzed and compared to reveal the relationship between PD-L1 expression heterogeneity and effective characteristics. This study explains the association of the PD-L1 22C3 CPS with the clinicopathological features and the heterogeneity of PD-L1 expression in EC in the Chinese population. We hope to find more evidence to reveal which specific population of EC patients are likely to obtain better benefits from immunotherapy.

METHODS

Patient study

A total of 533 surgical resection specimens from individuals with esophageal carcinoma (EC) were obtained from the pathology database from January 2019 through September 2020 at the Shanghai Cancer Center, Fudan University. The enrollment criteria are listed as follows. All 576 cases from January 2019 through September 2020 were enrolled, and we then excluded 43 cases for the reasons as follows: (1) The cases had no invasive lesion or not enough lesion for PD-L1 staining and scoring (31 cases), including after ESD treatment (8 cases), after neoadjuvant chemotherapy (NACT) (17 cases), and carcinoma in situ (6 cases). (2) The cases had no entire information (12 cases). Finally, we enrolled 533 cases. All these cases were enrolled based on a histological classification according to the 2019 World Health Organization classification, including squamous cell carcinoma, adenocarcinoma, basaloid squamous carcinoma, spindle cell carcinoma and neuroendocrine tumors.[27] Clinicopathological parameters were retrieved and collected from the medical records. The pathological stage was determined by the AJCC staging system (eighth edition).[28] A total of 37 paired paraffin blocks from the same resected samples and 50 paired primary tumor and metastatic lymph node samples were selected for the study. The 37 paired paraffin blocks were enrolled when the cases had two different paraffin blocks and contained enough tumor area available for PD-L1 assay. The 50 paired primary tumor and metastatic lymph node squamous cell carcinoma samples were enrolled when the cases had positive metastatic lymph nodes and the metastatic lesion contained enough tumor area available for PD-L1 assay. This study was approved by the Institutional Review Board (IRB, 050432-4-1911D, 2019) of Fudan University Shanghai Cancer Center.

Histological sample processing and PD-L1 immunohistochemical staining

Immunohistochemical analysis for PD-L1 expression was performed by using the PD-L1 IHC 22C3 pharmDx assay (Agilent Technologies) on a representative tumor section. The "representative" tumor section mentioned was selected by two experienced pathologists (achieve certification by

Targo training and become trainer in PD-L1 scoring training in China) before PD-L1 staining. The block was selected to have enough tumor region for PD-L1 staining, without or just with a small amount of macroscopic necrosis. This assay was performed on the Dako Autostainer Link 48 platform according to an automated staining protocol. The specific steps of PD-L1 IHC staining were performed as described previously.[29,30] Briefly, we followed the manufacturer's protocol for the Dako system and used the Dako clone 22C3 assay on the Dako Link 48 automated platform. Each analysis of PD-L1 was developed with both paired Dako positive and negative controls (cell line). In addition, we detected a positive PD-L1 tissue control (tonsil tissue) on every PD-L1 IHC-stained slide.

PD-L1 expression was evaluated by the CPS, which is defined as the number of PD-L1-stained cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells multiplied by 100. The maximum CPS is defined as 100. All other cells, such as tumor-associated plasma cells, neutrophils, normal/non-neoplastic cells, and necrotic cells, were excluded from the evaluation. The cutoff value was determined according to an FDA-approved test

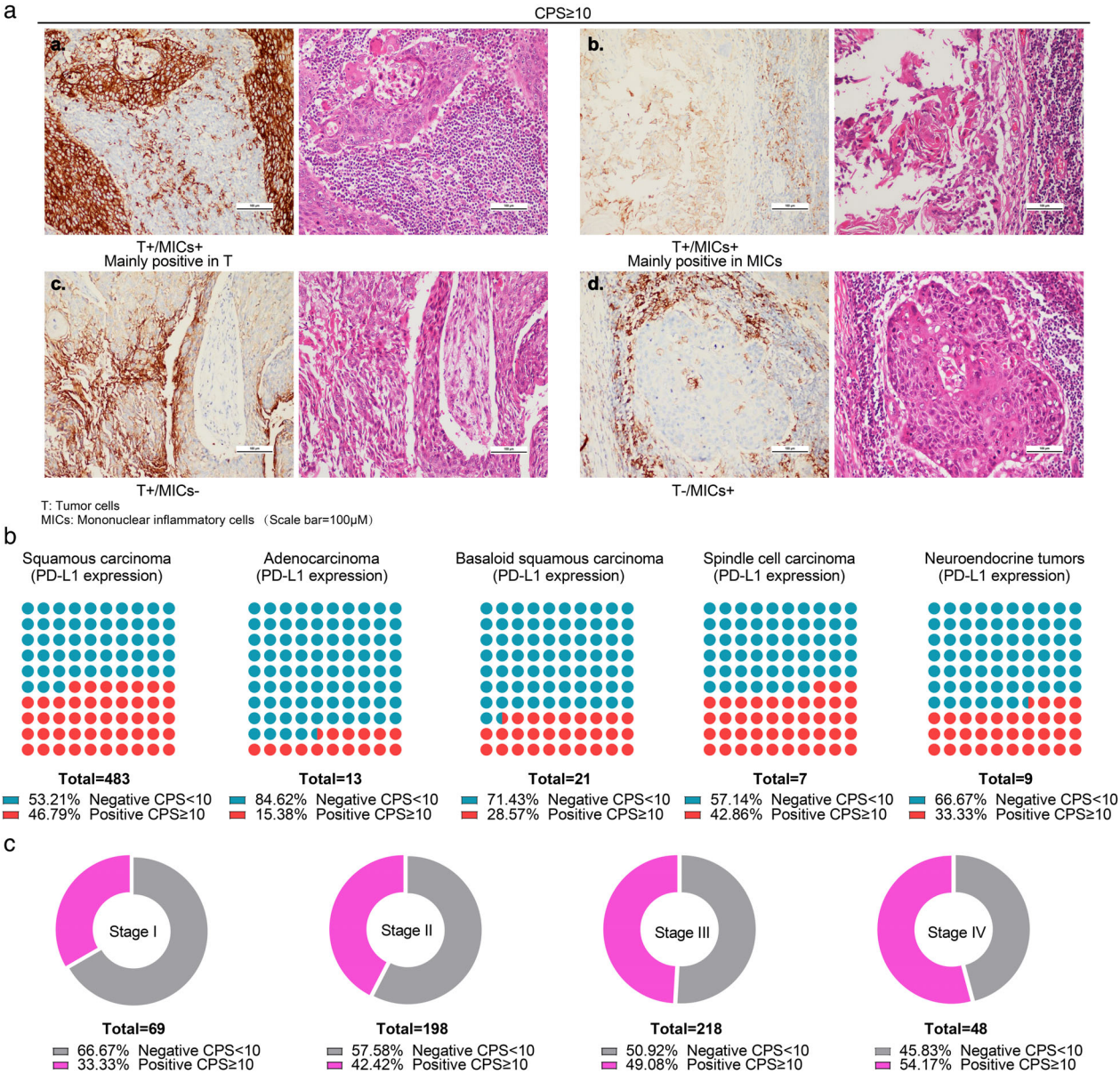

FIGURE 1 Clinicopathological analysis of cases with positive PD-L1 expression. (a) PD-L1 expression was determined by using CPS, which includes positive staining located in both tumor cells (T) and mononuclear inflammatory cells (MICs), only T cells, or only MICs. (b) PD-L1-positive rates were compared among different histological types, including squamous carcinoma (46.8%, 226/483), adenocarcinoma (15.4%, 2/13), basaloid squamous carcinoma (28.6%, 6/21), spindle cell carcinoma (42.9%, 3/7) and neuroendocrine tumors (33.3%, 3/9). The red dots represent positive PD-L1 22C3 CPS cases while blue dots represent negative PD-L1 22C3 CPS ones. (c) PD-L1-positive rates are listed in different pathological stages of EC [positive rate: I, 33.3% (23/69), II, 42.4% (84/198), III, 49.1% (107/218), IV, 54.2% (26/48), $p = 0.06$]

TABLE 1 PD-L1 CPS score and clinical parameters of patients

		PD-L1 expression (CPS)		
	Total ($n = 533$)	**Negative <10 ($n = 293$)**	**Positive ≥ 10 ($n = 240$)**	**p[a]**
Age				
Median (IQR[b])	65 (59–70)	65 (59–70)	65 (60–70)	
≤65	265 (49.7)	142 (48.5)	123 (51.3)	0.651
>65	268 (50.3)	151 (51.5)	117 (48.8)	
Sex				
Female	100 (18.8)	57 (19.5)	43 (17.9)	0.651
Male	433 (81.2)	236 (80.5)	197 (82.1)	
Tumor size				
<2.5 (25th percentile)	132 (24.8)	78 (26.6)	54 (22.5)	0.677
≥2.5 and <3.2 (50th percentile)	134 (25.1)	72 (24.6)	62 (25.8)	
≥3.2 and <4.5 (75th percentile)	132 (24.8)	73 (24.9)	59 (24.6)	
>4.5	135 (25.3)	70 (23.9)	65 (27.1)	
Histology				
Squamous carcinoma	483 (90.6)	257 (87.7)	226 (94.2)	0.082
Basaloid squamous carcinoma	21 (3.9)	15 (5.1)	6 (2.5)	
Adenocarcinoma	13 (2.4)	11 (3.8)	2 (0.8)	
Spindle cell carcinoma	7 (1.3)	4 (1.4)	3 (1.3)	
Neuroendocrine tumors	9 (1.7)	6 (2.0)	3 (1.3)	
Differentiation				
Well differentiated	42 (7.9)	22 (7.5)	20 (8.3)	0.846
Moderately differentiated	289 (54.2)	162 (55.3)	127 (52.9)	
Poorly differentiated	202 (37.9)	109 (37.2)	93 (38.8)	
NACT[c]				
Absent	490 (91.9)	266 (90.8)	224 (93.3)	0.282
Present	43 (8.1)	27 (9.2)	16 (6.7)	
Stage				
I	69 (12.9)	46 (15.7)	23 (9.6)	0.060
II	198 (37.1)	114 (38.9)	84 (35.0)	
III	218 (40.9)	111 (37.9)	107 (44.6)	
IV	48 (9.0)	22 (7.5)	26 (10.8)	
TNM_T stage				
T1	97 (18.2)	64 (21.8)	33 (13.8)	0.031
T2	104 (19.5)	62 (21.2)	42 (17.5)	
T3	329 (61.7)	165 (56.3)	164 (68.3)	
T4	3 (0.6)	2 (0.7)	1 (0.4)	
TNM_N stage				
N0	247 (46.3)	149 (50.9)	98 (40.8)	0.086
N1	151 (28.3)	75 (25.6)	76 (31.7)	
N2	88 (16.5)	48 (16.4)	40 (16.7)	
N3	47 (8.8)	21 (7.2)	26 (10.8)	
TNM_M stage				
M0	529 (99.2)	290 (99.0)	239 (99.6)	0.761
M1	4 (0.8)	3 (1.0)	1 (0.4)	
Lymph node metastasis				
Absent	247 (46.3)	149 (50.9)	98 (40.8)	0.021

(Continues)

TABLE 1 (Continued)

	Total ($n = 533$)	PD-L1 expression (CPS)		p^a
		Negative <10 ($n = 293$)	Positive ≥ 10 ($n = 240$)	
Present	286 (53.7)	144 (49.1)	142 (59.2)	
Venous/lymphatic invasion				
Absent	207 (38.8)	126 (43.0)	81 (33.8)	0.029
Present	326 (61.2)	167 (57.0)	159 (66.3)	
Perineural invasion				
Absent	353 (66.2)	202 (68.9)	151 (62.9)	0.143
Present	180 (33.8)	91 (31.1)	89 (37.1)	

[a]Chi-square test.
[b]IQR, interquartile range.
[c]NACT, neoadjuvant chemotherapy.

TABLE 2 The heterogeneity of PD-L1 expression between different blocks

	B block (PD-L1 expression)					
	Negative CPS < 10	Positive CPS ≥ 10	Total ($n = 37$)	Kappa value	p^a	p^b
A block (PD-L1 expression)						
Negative CPS < 10	11	3	14	0.7087	0.000016	2.6E-05
Positive CPS ≥ 10	2	21	23			
Total ($n = 37$)	13	24	37			

[a]Kappa statistic: approximate significance.
[b]Fisher's exact test.

and the guidelines of pembrolizumab treatment and separated into two classifications: negative (CPS < 10) and positive expression (CPS ≥ 10).[9,10,31] Patients without sufficient viable tumor cells (<100) were excluded. Each slide was blindly given a CPS for PD-L1 expression by two experienced pathologists. Both hematoxylin–eosin (HE) staining and PD-L1 IHC staining were assessed to reach a final CPS value. The evaluation of pathological slides was performed by two experienced pathologists (who had achieved Targo training certification and become a trainer in PD-L1 scoring training in China; each case has a final consistent result after discussion).

Statistical analysis

Statistical analyses were performed using the software package Statistical Package for Social Sciences, version 20.0, for Windows (SPSS). Chi-square or Fisher's exact tests were used to identify the influence of clinicopathological parameters on PD-L1 CPS values. Kappa tests were used to analyze the concordance between paired specimens, and the strength of concordance was categorized as follows: kappa value >0.75, perfect agreement; 0.4 to 0.75, moderate agreement; and <0.4, poor agreement. Pearson's correlation coefficient was calculated for the paired two clone CPS results of the samples. All statistical values were determined using two-tailed statistical analyses, and a p-value <0.05 was considered statistically significant.

RESULTS

Clinicopathological factors associated with PD-L1 expression in surgically resected specimens from EC patients

In total, 533 EC cases were eligible for our study; 240 (45%) patients had positive PD-L1 expression scores (CPS ≥ 10), while 293 (55%) patients had negative PD-L1 expression (CPS < 10). PD-L1 CPS-positive cases showed PD-L1 expression on tumor cells (T) and mononuclear inflammatory cells (MICs), only tumor cells, or only MICs (Figure 1(a)). Among these 533 EC patients, PD-L1-positive expression was more frequently observed in squamous cell carcinoma (46.8%, 226/483) than in other histological types, such as adenocarcinoma (15.4%, 2/13), basaloid squamous carcinoma (28.6%, 6/21), spindle cell carcinoma (42.9%, 3/7) and neuroendocrine tumors (33.3%, 3/9) (Figure 1(b)). The patients' clinicopathological characteristics are described in Table 1. PD-L1 positive expression was strongly associated with the presence of lymph node metastasis (142/240, 59.2%, $p = 0.021$) and venous/lymphatic invasion (159/240, 66.3%, $p = 0.029$). There was a similar trend for the PD-L1-positive proportion, but no

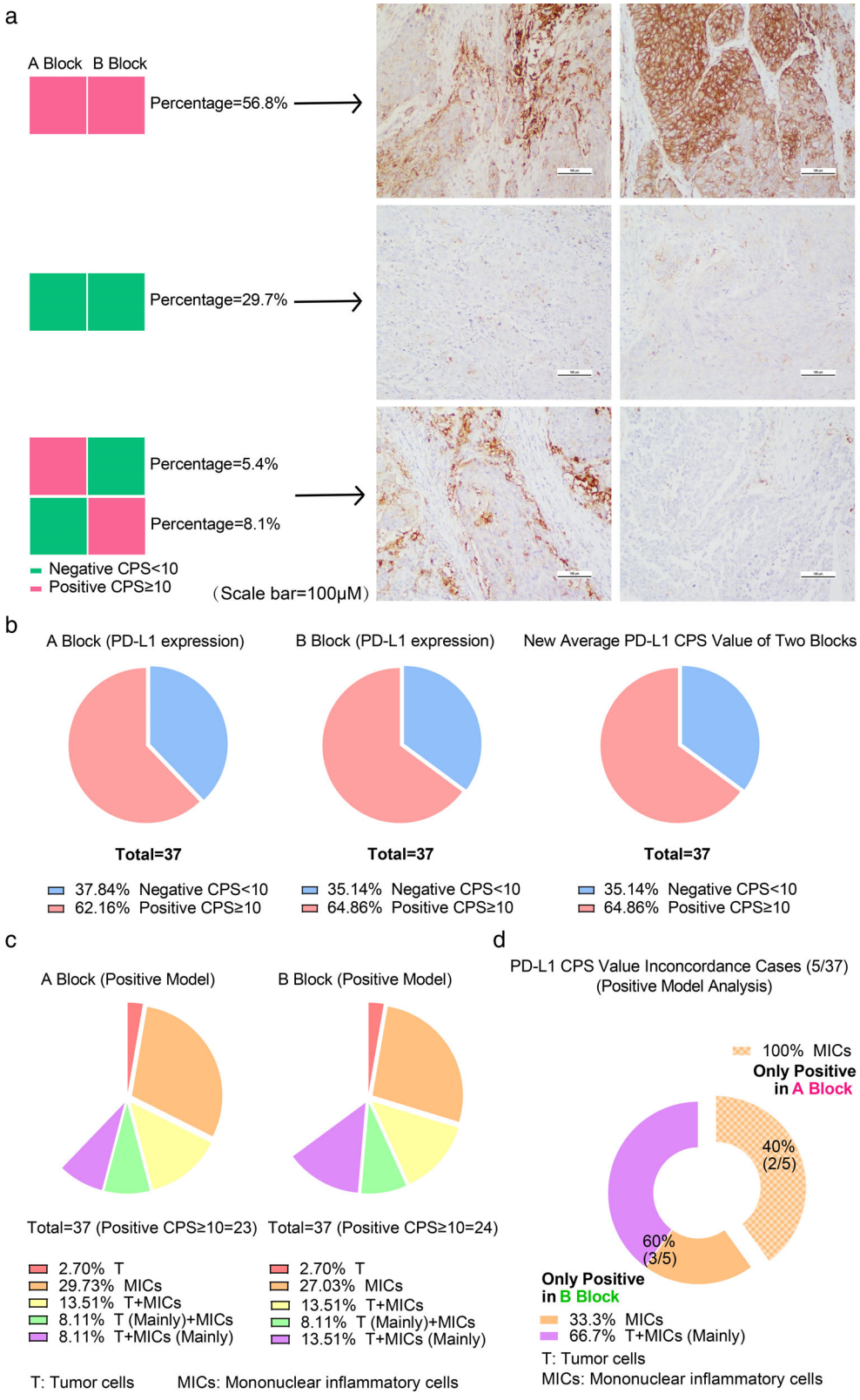

FIGURE 2 Frequency distribution of PD-L1 expression between different blocks. (a) PD-L1 expression in two different blocks, left one is A block and right one is B block. As shown in the figure, the rate of PD-L1 positive expression (CPS≥10) in both A and B blocks was 56.8%, while negative expression (CPS < 10) was 29.7%. The rate of PD-L1 positive expression in only A block was 5.4%, while in only B block was 8.1%. (b) High concordance was observed in the comparison of the CPS value between two paired blocks and their average value, with positive rate 62.16% in A block, 64.86% in B block, 64.86% for the average value of two blocks. (c) Similar PD-L1-positive cell types are displayed in the consistent cases. For A block, the PD-L1 positive model distribution is 2.70% of tumor cells (T), 29.73% of mononuclear inflammatory cells (MICs), 13.51% of T + MICs, 8.11% of T (mainly) + MICs, 8.11% of T + MICs (mainly). While for B block, the PD-L1 positive model distribution is 2.70% of tumor cells (T), 27.03% of mononuclear inflammatory cells (MICs), 13.51% of T + MICs, 8.11% of T (mainly) + MICs, 13.51% of T + MICs (mainly). (d) PD-L1-positive cell types are summarized in the heterogeneous cases. The results showed that only MICs (100.0%) were found in block A single positive cases, while both MICs (33.3%) and T + MICs (mainly) (66.7%) were found in block B single positive cases

statistical significance was found in different pathological stages (positive rate: I, 33.3% [23/69], II, 42.4% [84/198], III, 49.1% [107/218], IV, 54.2% [26/48], $p = 0.06$) (Figure 1(c)). We also observed a weak increase of PD-L1-positive rate in T stage (positive rate: T1, 34.0% (33/97), T2, 40.4% (42/104), T3, 49.8% (164/329), T4, 33.3% (1/3), $p = 0.031$). In addition, there were no significant correlations between PD-L1 expression variability and age, sex, tumor size, differentiation, N stage, M stage, status of neoadjuvant chemotherapy (NACT) or perineural invasion (Table 1).

TABLE 3 The heterogeneity of PD-L1 expression between primary and matched lymph node metastatic lesions

	Lymph node metastasis (PD-L1 expression)					
	Negative CPS < 10	Positive CPS ≥ 10	Total ($n = 50$)	Kappa value	p^a	p^b
Primary lesion (PD-L1 expression)						
Negative CPS < 10	11	6	17	0.5027	0.000373	0.000373
Positive CPS ≥ 10	5	28	33			
Total ($n = 50$)	16	34	50			

[a]Kappa statistic: approximate significance.
[b]Pearson's Chi-square test.

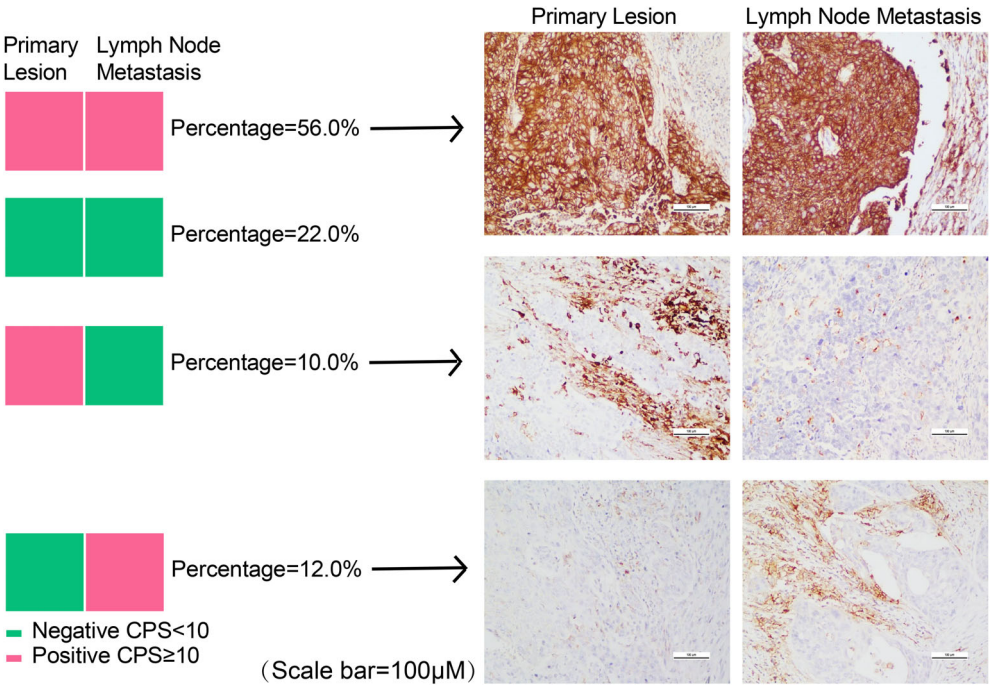

FIGURE 3 Frequency distribution of 50 PD-L1 expression between paired primary tumors and metastatic lymph node lesions. As shown in the figure, the rate of PD-L1 positive expression (CPS ≥ 10) in both primary and metastatic lymph node lesions was 56.0%, while negative expression (CPS < 10) was 22.0%. The rate of PD-L1 positive expression in only primary lesion was 10.0%, while in only metastatic lymph node lesion was 12.0%

Heterogeneity of PD-L1 expression between different blocks

A total of 37 paired paraffin blocks from the same resected squamous cell carcinoma sample were selected from the 533 ECs to identify the heterogeneity between different blocks in the same case (block A and block B). The concordance rate of the PD-L1 CPS between two different blocks was 86.5% (32/37), with a kappa value of 0.7087 (high consistency, $p = 0.000016$) (Table 2). The PD-L1 CPS values were variable between different paraffin blocks in 5 (13.5%) of 37 cases (Table 2 and Figure 2(a)). Interestingly, the new average CPS value of two different blocks was completely coordinated with the PD-L1 expression value of a single A or B block (Figure 2(b)). Further analysis was performed on the different proportions of positive cells of five heterogeneous cases, and the data indicated that the MICs were responsible for the inconsistency in PD-L1 expression between different blocks. For cases where the PD-L1 CPS was positive only in block A, 100% (2/2) of the cases showed PD-L1 expression on MICs. For other cases in which the PD-L1 expression was only positive in B block, 33.3% (1/3) of the cases showed PD-L1 expression on MICs and 66.7% (2/3) on both tumor cells (T) and mononuclear inflammatory cells (MICs), especially MICs, which are called T + MICs (mainly) (Figure 2(c),(d)).

Heterogeneity of PD-L1 expression between paired primary and metastatic lymph node lesions

A total of 50 paired primary squamous cell carcinoma samples and metastatic lymph nodes were selected from the 533 EC samples to identify the heterogeneity of PD-L1 expression between paired primary tumors and metastatic lymph nodes. The rates of PD-L1 negative expression (CPS < 10) and positive expression (CPS ≥ 10) were 34% (17/50) and 66% (33/50) in the primary tumors, respectively. In the paired metastatic lymph node lesions, the rates of PD-L1 expression changed to 32% (16/50) for negative cases and 68% (34/50) for positive cases. Among all these 50 cases, only 22% were PD-L1 only positive in either

primary lesion or metastatic lymph node lesion cases. Most samples (78.0%) showed a consistent expression of PD-L1 in two lesions. (Table 3 and Figure 3). As shown in Table 3, the PD-L1 CPS value showed an agreement rate of 78% (39/50) between the primary tumors and metastatic lymph node lesions, with a kappa value of 0.5027 (moderate agreement, $p = 0.000373$). Among the 22% (11/50) of discordant cases, 45.5% (5/11) were positive in primary lesions, whereas 54.5% (6/11) were positive in metastatic lymph nodes.

DISCUSSION

Despite recent advances in the treatment of esophageal cancer with the addition of targeted therapy to chemotherapy, the incremental survival benefits of these drugs are only a few months, and the overall survival of patients remains relatively poor.[1–3,5–7] After the immune checkpoint inhibitor pembrolizumab was approved by the FDA, the poor prognosis of advanced cancer patients has been reported to improve.[24,25] The use of immune checkpoint inhibitors emphasizes the need for comprehensive scoring of PD-L1 expression, which helps better identify candidates that respond well to immunotherapy. The PD-L1 22C3 CPS currently has FDA approval as a companion diagnostic (CDx) for immunotherapies in esophageal cancer in 2019. PD-L1 22C3 is certified as a companion diagnostic test (CDx) for pembrolizumab, and the PD-L1 22C3 CPS scoring algorithm defines PD-L1 positivity in esophageal cancer. Previous studies have determined that PD-L1 expression occurs in approximately 40% of esophageal cancers.[14,15] However, the problems posed by the different assays/antibodies and scoring systems used to assess PD-L1 status are well known. Currently, little is known about the relationship between PD-L1 22C3 CPS and the clinicopathological characteristics of EC.

To our knowledge, this study represents a novel and the largest comprehensive study of PD-L1 22C3 CPS with clinicopathological characteristics in Chinese patients with EC. The findings of the current study are as follows: (1) PD-L1 positive expression was more frequently observed in squamous cell carcinoma than other types. (2) PD-L1 positive expression was strongly associated with the presence of lymph node metastasis and venous/lymphatic invasion. (3) A similar trend of the PD-L1-positive proportion with advanced pathological stages were observed but lacked statistical significance. (4) PD-L1 expression has a high consistency in different paraffin blocks of the same surgically resected specimen and paired primary/metastatic lymph node lesions.

These results demonstrate high PD-L1 expression in advanced patients, especially those with a risk of metastasis, and these advanced EC patients are likely to obtain better benefits from immunotherapy. However, this positive association of PD-L1 expression with "lymph node metastasis" and "venous/lymphatic invasion" (short for "invasion/metastasis" in the manuscript) was not dramatically high but still around 60%, (59.2% and 66.3%, respectively). Also, the trend of correlation with pathological stage is somewhat challenging since it was not statistically significant and the range of positive correlation among stages I–IV was narrow (33.33%–54.17%). For T stage, there was a weak increase of PD-L1 positive rate from T1 to T3, but the number for T4 was too low to reach a solid conclusion whether the correlation is real. Also, no significant difference was shown between the variability in PD-L1 expression and age, sex, tumor size, different histopathological subtypes, differentiation, N stage, M stage, status of neoadjuvant chemotherapy (NACT) or perineural invasion. Obviously, the invasion/metastasis of tumor cells contribute most to affect PD-L1 expression. It indicates that the biomarkers involved in aggravating invasion and metastasis might be important for PD-L1 expression in EC. Rong et al. also showed that PD-L1 SP142 expression was significantly related to stage and metastasis, which was consistent with our findings.[14]

Previous studies reported the high RNA level of the CD274 gene (PD-L1) in focal advanced EC patient cells[24,25] and compared PD-L1 SP142 expression only in adenocarcinoma between primary tumors and metastases by tissue-array,[26] but lacked a detailed discussion of PD-L1 protein heterogeneity in EC. The inconsistency in PD-L1 expression in different paraffin blocks of the same surgically-resected specimen was mainly related to infiltrated positive mononuclear inflammatory cells (MICs). More interestingly, the new average CPS value calculated by two paired blocks also had a high concordance with the individual PD-L1 expression of both blocks A and B. The variability was not remarkable in different tumor blocks. It is common that pathologists have the random selection on blocks for PD-L1 CPS evaluation in routine work. The significant concordance in the new average CPS value might implicate that there is limited effect on the final CPS in most cases with different blocks. The "average CPS values" are worthy of being applied in the heterogenous CPS cases. In addition, the five heterogeneous cases all had both lymph node metastasis and venous/lymphatic invasion, but no clear evidence was found to support the involvement of invasion/metastasis in this difference. It is therefore hard to reach a specific conclusion about why PD-L1 positive expression is strongly associated with lymph node metastasis and venous/lymphatic invasion. However, this finding is scientific evidence and might be useful for the potential application of immune checkpoint inhibitors on advanced esophageal carcinoma patients, especially those with a metastatic burden. Analysis of different proportions of positive cells indicated that the inconsistency in PD-L1 expression was related to the number of positive MICs. In the future, we could combine immunochemical staining results of CD4 and CD8 to better determine the effect of different types of mononuclear inflammatory cells on PD-L1 expression heterogeneity. In contrast to different paired blocks, there was significantly less consistency in the primary tumor and metastatic lesions. This finding likewise underlies a potential link between inconsistent PD-L1 expression and invasion/metastasis.

Indeed, we also have some limitations that could be further addressed, especially the relationship between heterogeneity and invasion/metastasis risk. As all our enrolled cases for the PD-L1 heterogeneity study had complete follow-up information, we have some short-term survival analysis on the progression-free survival (PFS) percentage between homogeneous and heterogeneous CPS cases (the deadline of follow-up is on date 2021/11/7). For two different blocks, an apparent tendency has been observed that the homogeneous CPS cases have lower PFS percentage compared to the heterogeneous ones. Although the statistical analysis is not significant (data not shown here), the difference is still valuable to follow up until we have enough numbers and a long-term observation. In addition, a more detailed analysis is needed to determine whether invasion/metastasis-related genes contribute to MIC infiltration and PD-L1 expression, which will be our next step. More samples should be obtained in order to reach a more solid conclusion. We also observed a trend of PD-L1 expression associated with histological types which could be the result of limited case selection. In our study population, SCC obviously occupied most of the total cases, while other types only had a low incidence. Although this represents a real Chinese EC population,[32] an uncertain conclusion of correlation analysis would also be reached. It is better to perform further research on both SCC population only or more cases of different histological types. Further, we found the heterogeneity in PD-L1 expression is due to MICs within the sample. However, we did not distinguish if the MICs were TAMs, DCs, B-cells or CD4/CD8+ T cells. What we believe is that the detail portion of MICs will help study which contributes most to PD-L1 positive expression. This is also our aim in future studies Finally, we simply compared the "average CPS value" which is the average value of two blocks to either single block. Although the consistency is easily seen in Figure 2(b), two blocks did not completely reflect the whole tumor lesion. More cases and more blocks therefore need to be observed.

Overall, this novel study demonstrated a positive correlation between high PD-L1 22C3 expression and invasion/metastasis risk in a cohort of 533 esophageal cancer patients. A significant consistency was revealed between both paired blocks and paired primary/metastatic lymph node lesions. The infiltrated MICs and invasion/metastasis risk are deduced to be responsible for PD-L1 heterogeneity, which might have substantial implications for clinical practice.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Ziling Huang https://orcid.org/0000-0002-0899-1270
Haiquan Chen https://orcid.org/0000-0001-7846-257X

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