Tumour Marker Prognostic Study

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Patterns and prognostic values of programmed cell death-ligand 1 expression and CD8$^+$ T-cell infiltration in small cell carcinoma of the esophagus: a retrospective analysis of 34 years of National Cancer Center data in China

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Background: Small cell carcinoma of the esophagus (SCCE) is an extremely rare and highly aggressive neuroendocrine malignancy with a strikingly poor prognosis. Given the great clinical successes of checkpoint immunotherapies, we explored the expression profile and clinical significance of programmed cell death-ligand 1 (PD-L1) and CD8$^+$ T cell in SCCE for the first time.

Materials and methods: Tumor-infiltrating immune cells (TIICs) and tumor cells in postoperative, whole tumor sections from 147 SCCE patients were stained for PD-LI expression. We also evaluated each patient's Combined Positive Score (CPS). Multiplex immunofluorescence staining (CD3, CD20, CD68, and PD-L1) was introduced to clarify the location of PD-L1. CD8 density was analyzed by digital imaging and analysis of entire slides. Clinical outcomes were tested for correlations with both PD-L1 expression and CD8 density.

Results: No patients had PD-L1 expressed in their tumor cells. PD-L1$^+$ expression in TIICs was detected in 65 patients (44.2%) and 42 (28.6%) exhibited CPS positivity. Multiplex immunofluorescence staining demonstrated that most of the PD-L1 was expressed on the CD68$^+$ monocytes/macrophages. PD-L1 expression in the TIICs and CPS was found to be correlated with paraffin block age, tumor length, macroscopic type, T stage, and increased overall survival (OS). Expression of PD-L1 in TIICs showed significantly prolonged relapse-free survival (RFS). Increasing CD8 densities were associated with increased PD-L1 expression ($P_{trend} < 0.0001$). Multivariate regression confirmed that PD-L1 in TIICs and CD8 states were independent predictors of OS, and CD8 status were found to be independently predictive of RFS. A stratification based on PD-L1 and CD8 status was also significantly associated with both OS and RFS.

Conclusion: Expression of PD-L1 was only detected in TIICs from approximately half of the patients with SCCEs. In SCCEs, PD-L1 and CD8 status are novel prognostic biomarkers and may inform the implementation of risk-related therapeutic strategies. SCCEs with higher CD8 infiltration also had higher expression of PD-L1, suggesting the development of resistance against adaptive immunity. These findings support the assertion that PD-L1/programmed cell death 1 inhibitors should be investigated in this rare malignancy.

Keywords: CD8, immunotherapy, programmed cell death-ligand 1, small cell carcinoma of the esophagus, tumor biomarkers

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SCCE is a sporadic and rather aggressive neuroendocrine gastrointestinal malignancy. Its incidence constitutes 0.8–2.4% of all histologic types of esophageal malignancies globally[1,2]. SCCE is characterized by early lymph node invasion, distant metastases, and poor prognosis[3]. Unfortunately, patients are often diagnosed with advanced-stage SCCE, and the median survival is only 8–13 months[4,5]. Although the first case of SCCE was reported in 1952, there is no standard treatment because of its low incidence and few cases[6]. Given its histologic resemblance to small cell lung carcinoma (SCLC), current treatment recommendations were extrapolated from well-established therapeutic strategies for SCLC. Most patients with SCCE experience recurrence within 1 year of initial treatment and die within a few months, even those who undergo aggressive initial therapy[7], thus necessitating novel and effective therapeutic approaches to improve outcomes.

Our understanding of the immune landscape of cancers has deepened over the last decade. The immune surveillance evasion process[8,9]has allowed the development of a new class of agents. The advent of treatment strategies to enable stronger immune responses targeted against tumors – especially monoclonal antibodies (mAbs) inhibiting immunoregulatory checkpoint proteins including programmed cell death 1 (PD-1) as well as PD-L1 – has offered inspiring new treatments for patients with cancer[10,11]. Recently, the Food and Drug Administration (FDA) approved atezolizumab (anti-PD-L1 antibody) as first-line treatment and nivolumab and pembrolizumab (anti-PD-1 mAbs) as third-line monotherapies for patients with SCLC[12–14]. These approvals were a significant milestone for patients with neuroendocrine malignancies – whose therapeutic strategies and clinical outcomes were unchanged for decades – and brought hope to patients with SCCE. However, as the most common extrapulmonary small cell carcinoma[15], no study has examined the relationship between ICIs that disrupt the interaction of PD-1 and PD-L1 and SCCE. Therefore, the application of this approach may help improve the treatment of SCCE.

ICIs are believed to be more effective in patients whose preexisting tumor-infiltrating lymphocytes (TILs) are responsive to activation of PD-L1, and can thus be re-activated by treatment with anticheckpoint antibodies[10,16–19]. In addition, it has been suggested that PD-L1 expression in the TC membrane or/and TIICs is correlated with improved outcomes after treatment with anticheckpoint mAbs such as anti-PD-1/PD-L1 immunotherapy in various malignancies, including SCLC[20,21] Emerging evidence has also suggested that the density of TILs – especially $CD8^+$ TILs – in the tumor region also helped determine the potential response to ICIs[22,23]. Thus, an improved understanding of the role of PD-L1 expression patterns and the extent of $CD8^+$ T-cell infiltration in SCCE is fundamental to the successful application of ICIs and may thus have considerable clinical implications.

Given the recent breakthrough successes of checkpoint therapies such as PD-L1 and PD-1, in this study, we assessed expression of PD-L1 and infiltration of $CD8^+$ T cells using immunohistochemistry (IHC) and multiplex immunofluorescence staining and investigated their association with clinical outcomes in a large cohort of tissue samples taken from patients with SCCE. This is the first and most comprehensive study of expression patterns and prognostic features of PD-L1 and $CD8^+$ T-cell density in patients with previously untreated, resected SCCE.

HIGHLIHGTS

- Small cell carcinoma of the esophagus (SCCE) is a rare and aggressive malignancy with a poor prognosis and no standardized treatment.
- Understanding the expression pattern of programmed cell death-ligand 1 (PD-L1) and CD8 is fundamental to the application of immune-checkpoint inhibitors (ICIs).
- PD-L1 was not observed in tumor cells (TCs) but appeared in tumor-infiltrating immune cells (TIICs) in 44.2% of SCCE specimens.
- PD-L1 expression and CD8 status were novel independent prognostic predictors of SCCEs.
- CD8 density was positively related to PD-L1 expression, supporting further investigation of ICIs in SCCEs.

Materials and methods

Study patients and tissue samples

We conducted a retrospective analysis of 147 resected tissue samples from patients with previously untreated SCCE treated at the National Cancer Center from 1985 to 2019. This study was evaluated and approved by our institutional ethics committee, who determined that this study did not require informed consent because it was a retrospective analysis. The work is fully compliant with the STROCSS 2021 criteria[24] (Supplemental Digital Content 5, http://links.lww.com/JS9/A9). All data were anonymously analyzed and reported in aggregate.

The pathologic diagnosis of SCCE was performed based on the histologic criteria from the 2010 WHO guidelines[25]. Hematoxylin and eosin–stained slides from each sample were independently assessed by two expert pathologists (L.Y.X. and Z.Y.Y.). Each case's diagnosis was further confirmed by detecting cytokeratin (pancytokeratin AE1/AE3) and neuroendocrine markers, including synaptophysin and chromogranin A. For cases with multiple histologic subtypes, the diagnosis of SCCE was made only if more than 70% of the cells met SCCE criteria. Routine physical examinations, including plain radiographs and chest computed tomography scans, found no evidence of other tumors, including SCLC, at diagnosis. Cases that underwent presurgical chemotherapy or radiotherapy were excluded from this study.

The clinicopathologic characteristics of all enrolled SCCEs were carefully reviewed and staged in line with the American Joint Committee on Cancer (seventh edition) system of classification. The tumor was restricted to a localized anatomic region in all patients, with the presence or absence of regional lymph node metastases. Relapse-free survival (RFS) was considered to be the time from esophagectomy to the date of recurrence, metastasis, or last contact. Overall survival (OS) was considered to be from the period between the esophagectomy and either the date of death or last contact. And the work has been reported in line with the REMARK criteria (Supplemental Digital Content 6, http://links.lww.com/JS9/A10).

Immunohistochemistry and multiplex immunofluorescence staining

For IHC, we prepared 4-μm formalin-fixed paraffin-embedded (FFPE) sections from large, resected specimens on glass slides for

staining. After deparaffinization and rehydration along a gradient of ethanol concentrations to distilled water, the slides were covered for 15 min with 3% H_2O_2 (Dako, Glostrup, Denmark) to block endogenous peroxidase activity. After that, antigen retrieval was carried out for 30 min in EDTA buffer (pH 9.0) using a microwave. The tissue sections were then blocked using 10% normal serum before incubation with the primary antibody. PD-L1 was stained overnight at 4°C using the E1L3N rabbit mAb (1:200, clone E1L3N, catalog 13684; Cell Signaling Technology, Danvers, Massachusetts, USA). For CD8 staining, a monoclonal mouse antibody (1:200, clone C8/144B, catalog M7103; Dako, Carpinteria, California, USA) was used overnight at 4°C. A secondary antibody, goat anti-mouse/rabbit-HRP (Dako, Glostrup, Denmark), was incubated for 30 min at room temperature. Finally, the staining signal was visualized using 3,3′-diaminobenzidine (DAB; Dako, Glostrup, Denmark), and hematoxylin was used for counterstaining. For multiplex immunofluorescence staining, tissue microarrays were constructed from three 1.0 mm cores of tumor tissues. Other three mAbs (CD3, 1:3000, clone D7A6E, catalog 85061; CD20, 1:3000, clone E7B7T, catalog 48750; CD68, 1:200, clone D4B9C, catalog 76437; Cell Signaling Technology) were used during the immunofluorescence staining, and the protocol was the same as previous study[26].

Quantification of programmed cell death-ligand 1 expression

PD-L1 staining was assessed by two expert pathologists (L.Y.X. and Z.Y.Y.) who were blinded to patient data and outcomes. Doubtful cases were discussed under a multiheaded microscope until consensus was achieved. TCs and TIICs were scored separately. All the sections featured at least 100 TCs. Positive expression of PD-L1 in TCs was defined as at least 1% of the TCs showing circumferential or partial membranous staining. Similarly, TIICs were considered PD-L1 positive if at least 1% of the immune cells (intratumoral and peritumoral) – including TILs and tumor-associated macrophages – were positive upon membranous or cytoplasmic staining. The peritumoral region was defined as an extension of 500 μm from the border and malignant nests to the host tissue[27]. Then, we also calculated a Combined Positive Score (CPS)[28], which was defined as the ratio of the total number of PD-L1-positive cells (tumor, TILs, and tumor-associated macrophages) to viable TCs, multiplied by 100. Similarly, CPS ≥ 1 was considered positive. Necrotic areas and only cytoplasmic TC staining were excluded from scoring.

Digital image acquisition and quantification of CD8 immunostaining

Digital image analysis was performed to quantify the $CD8^+$ TILs density (intratumoral and peritumoral) on the whole section slides stained with CD8 antibodies. All slides were first scanned at high resolution ($\times 400$) using a Panoramic MIDI II slide scanner from 3DHISTECH. A trained gastrointestinal pathologist (L.Y.X.) annotated the tumor regions using CaseViewer_2.3. Then, the HALO 'Membrane IHC Quantification' module was used to identify the number of $CD8^+$ cells in the created compartment and measure each compartment's exact area. Data output was the density of CD8 cells/mm^2, defined as the count of positively stained cells divided by the compartment's area.

Statistical analysis

SPSS 25.0 version (IBM Corporation, Chicago, IL, USA), SAS 9.2 version (SAS Institute, Cary, NC, USA), and R 3.5.1 version (Lucent Technologies, USA) were used to analyze and generate figures. We compared positive PD-L1 expression and increased levels of CD8 expression using the Cochran–Armitage test for trends. The extent of association between PD-L1 expression or $CD8^+$ TIL density and clinicopathologic characteristics was assessed with χ^2 or Fisher's exact tests, as appropriate. The mean densities were compared using the Wilcoxon rank-sum test. The Kaplan–Meier method (using the log-rank test) was used to calculate the probabilities of OS and RFS. We identified independent predictors of prognosis using multivariate analysis with a Cox proportional hazards model. A *P*-value (two-sided) less than 0.05 was set as the limit for statistical significance.

Results

Patient characteristics and clinical outcomes

The clinicopathologic data from the 147 patients with newly diagnosed primary SCCE who underwent surgical resection are summarized in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JS9/A2). All patients were at a limited stage of disease, and most were male (74.1%). Seventy-one patients (48.3%) had a history of alcohol abuse, and 54 patients (36.7%) had a smoking index greater than 400. The most common site of origin was the middle third of the thoracic esophagus (75.5%). Regarding the histologic type, 78.9% of the specimens were pure SCCE, the others were mixed-type. Ninety-seven cases (66.0%) with lymph node metastasis were detected among the 147 patients. No patients received preoperative neoadjuvant therapies, and 67.3% received adjuvant therapies after surgery. The median OS and RFS time of the 147 patients were 22 months [95% confidence interval (CI): 17.279–26.721] and 13 months (95% CI: 11.019–14.981), respectively. The 1-year, 3-year, and 5-year rates of OS and RFS were 75.0, 33.7, and 26.0%; and 52.4, 23.2, and 17.9%, respectively.

Programmed cell death-ligand 1 expression and clinicopathologic features

Measurement of PD-L1 protein expression via IHC was conducted using the anti-PD-L1 antibody E1L3N. Interestingly, none of the 147 tested SCCEs stained positive for PD-L1 in TCs (Fig. 1). Unlike the expression patterns in neoplastic cells, 44.2% (65/147) of the cases showed PD-L1 expression within TIICs, with a median value is 0 and mean ± SD is 9.041 ± 15.3 (Fig. 1 and Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JS9/A2). Considering that SCLCs with CPS ≥ 1 were found to be more likely to benefit from pembrolizumab treatment in a previous clinical trial (KEYNOTE-158)[29], PD-L1 expression in SCCEs was also evaluated by CPS. Using the criterion of CPS ≥ 1 as positive, 42 out of 147 cases of SCCEs were PD-L1 positive (28.6%), with median value is 0 and mean ± SD is 1.959 ± 4.748 (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JS9/A2). In addition, we also explored what kind of immune cells PD-L1 expressed on. Considering the T cells ($CD3^+$), B cells ($CD20^+$), and monocytes/macrophages ($CD68^+$) immune cells are the major components of the TIICs, multiplex immunofluorescence staining was

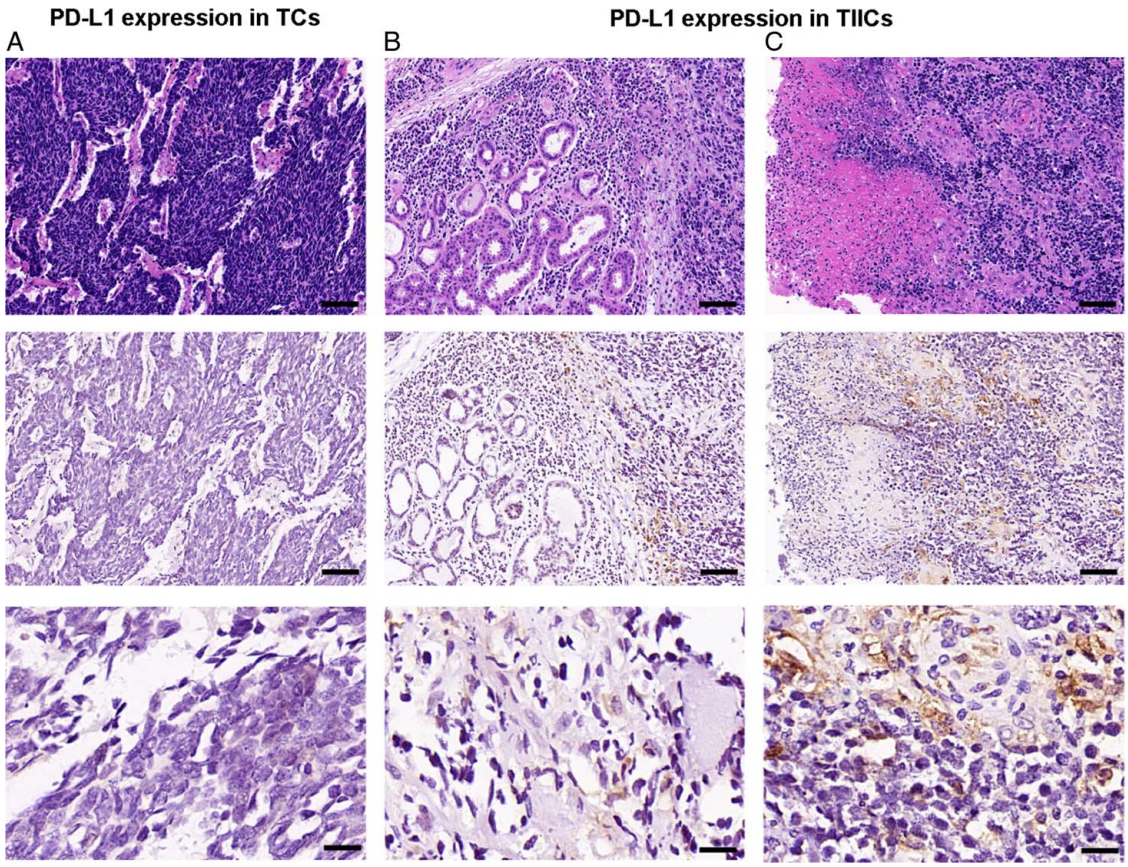

Figure 1. Expression of programmed cell death-ligand 1 (PD-L1) in small cell carcinoma of the esophagus. (A) Tumor cells (TCs) in small cell carcinoma of the esophagus did not show any PD-L1 staining. (B) One percent of the tumor-infiltrating immune cells (TIICs) showed PD-L1 positive. (C) Fifty percent of the TIICs showed PD-L1 positive. Hematoxylin–eosin staining (upper panel, ×100, scale bars, 100 μm) and PD-L1 staining (middle panel, ×100, scale bars, 100 μm; lower panel, ×400, scale bars, 20 μm) of small cell carcinoma of the esophagus.

introduced to clarify the co-expression of PD-L1 and these types of immune cells. As shown in Figure 2, results indicated that almost all the PD-L1 was located at the $CD68^+$ monocytes/macrophages, whereas rare to find PD-L1 expressed on the $CD3^+$ or $CD20^+$ TIICs.

The associations between expression of PD-L1 and clinical features are shown in Table 1. Notably, the apparent expression of PD-L1 in TIICs was actually found to be closely related to FFPE block age ($P < 0.001$), as well as BMI ($P = 0.049$), T stage ($P = 0.028$), macroscopic tumor type ($P = 0.011$), tumor length ($P = 0.003$), and cancer-specific mortality ($P = 0.004$). PD-L1-positive staining by CPS was found to be significantly associated with FFPE block age ($P < 0.001$), tumor length ($P = 0.014$), macroscopic tumor type ($P = 0.011$), as well as T stage ($P < 0.001$).

Infiltration of $CD8^+$ T cells and clinicopathologic features of small cell carcinoma of the esophagus

$CD8^+$ T cells play a central part in the adaptive immune response and can be inhibited by PD-L1 expression. As a result, their presence and characteristics are key factors determining the potential responsiveness of tumors to treatment with ICIs[30,31]. We quantified $CD8^+$ T-cell infiltration in SCCEs through analysis of whole slide images (Fig. 3a). The median number of $CD8^+$ T cells per slide was 111.1 cells/mm^2, with a range of 7.5–1458.6 cells/mm^2, and mean ± SD is 195.9 ± 272.1 cells/mm^2 (Supplementary Table 1, Supplemental Digital Content 1, http://links.lww.com/JS9/A2). Next, we assessed $CD8^+$ T-cell infiltration in SCCEs within other small cell carcinomas on different areas and esophageal cancer with different pathologic types[32]. With the mean number in SCCEs of 195.9 cells/mm^2, the $CD8^+$ T-cell infiltration status in SCCEs was very similar to that of SCLCs, with the mean number of 192 cells/mm^2 (Supplementary Fig. 1, Supplemental Digital Content 2, http://links.lww.com/JS9/A3). In SCCE samples, $CD8^+$ T-cell infiltration status more closely resembled esophageal squamous cell carcinoma (ESCC), with a mean number of 205 cells/mm^2, than esophageal adenocarcinoma with the mean number of 378 cells/mm^2 (Supplementary Fig. 1, Supplemental Digital Content 2, http://links.lww.com/JS9/A3).

The relationships between $CD8^+$ T-cell infiltration and clinicopathologic variables are summarized in Supplementary Table 2 (Supplemental Digital Content 3, http://links.lww.com/JS9/A4). The mean destiny (195.9 cells/mm^2) was used as a cutoff value stratifying $CD8^+$ T-positive or T-negative groups. Positive $CD8^+$ T-cell infiltration was significantly related to macroscopic tumor type ($P = 0.006$), T stage ($P = 0.007$), TNM stage ($P = 0.005$), as well as cancer-specific mortality ($P = 0.004$).

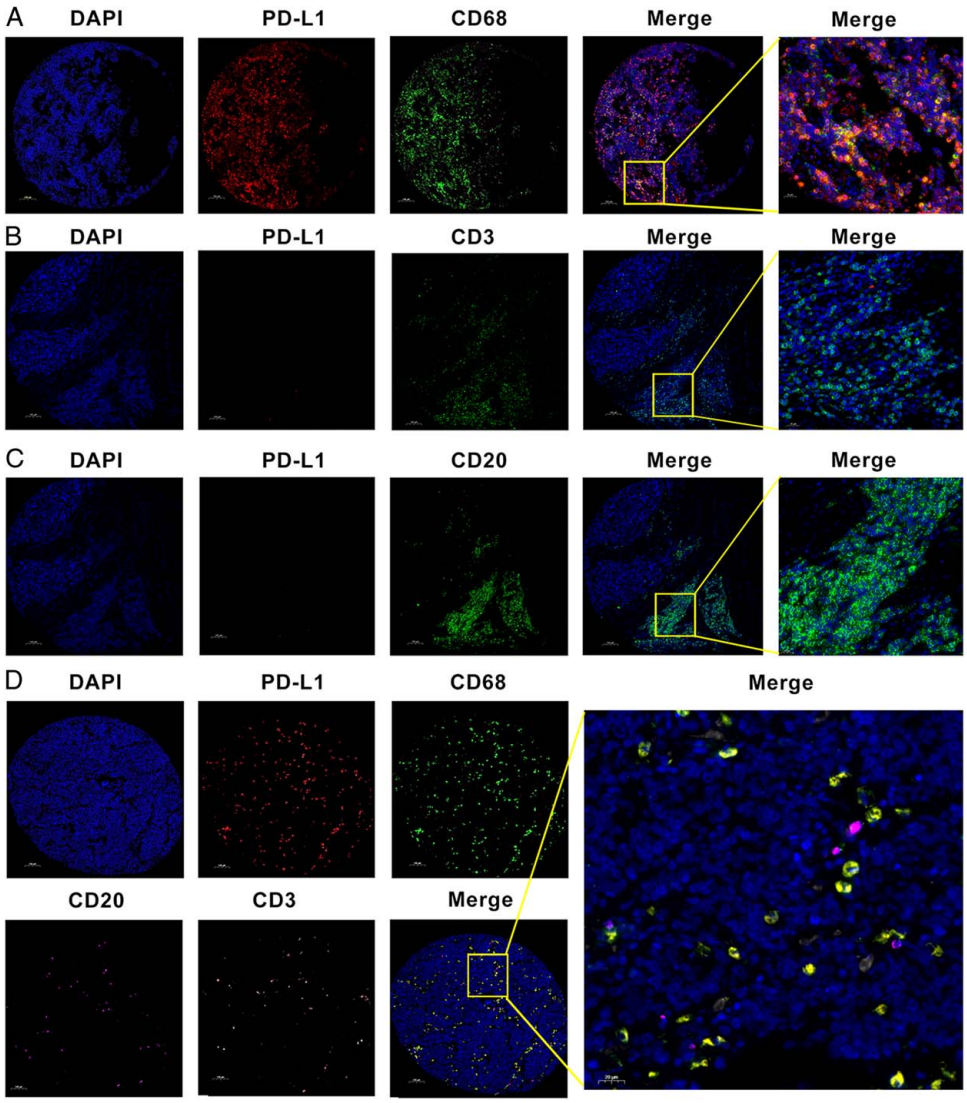

Figure 2. PD-L1 was mainly located on the CD68$^+$ tumor-infiltrating immune cells. Representative multiple immunofluorescence images of CD68/PD-L1 (A), CD3/PD-L1 (B), CD20/PD-L1 (C), and CD3/CD20/CD68/PD-L1 (D) from tissue microarrays. Left panel, × 100, scale bars, 100 μm; right panel, × 400, scale bars, 20 μm. PD-L1, programmed cell death-ligand 1.

Association between CD8$^+$ T-cell infiltration and expression of programmed cell death-ligand 1

Next, we evaluated for a potential association between the expression of PD-L1 and CD8$^+$ T-cell infiltration among patients with SCCEs. PD-L1 expressions in TIICs and the CPS were significantly associated with CD8$^+$ T-cell infiltration status with *P*-value of 0.001 and *P*-value less than 0.001, respectively (Table 1). As continuous values, PD-L1 expression in the TIICs and the CPS were significantly associated with CD8$^+$ T-cell infiltration status with *P*-values of 0.013 and 0.002, respectively (Supplementary Fig. 2, Supplemental Digital Content 2, http://links.lww.com/JS9/A3). The absolute counts of CD8$^+$ T cells in SCCEs with or without expression of PD-L1 were also explored. As shown in Figures 3b and 3c, CD8$^+$ T-cell density was found to be markedly higher in those who had PD-L1-positive tumors ($P < 0.05$).

To better assess the association between the CD8$^+$ T-cell density and PD-L1 expression, the density of CD8$^+$ T cells was stratified into levels by quartiles: low ($< 36.1/mm^2$), mid ($36.1–224.1/mm^2$), and high ($> 224.1/mm^2$). Then, the associations between CD8$^+$ T-cell density measurements and expression of PD-L1 in SCCEs were examined. As expected, increasing CD8$^+$ T-cell density was found to be correlated with increased PD-L1 expression in TIICs or by CPS (Fig. 3d). Only 13.89% PD-L1 positive in TIICs was found in samples with low CD8 density, whereas 46.67% and 69.44% PD-L1 positive in TIICs were found in patients with mid and high CD8 densities ($P_{trend} < 0.0001$). Likewise, PD-L1 positive by CPS was only found in 2.80% of the low CD8 group, while 29.33% (mid-CD8 density) and 54.29% (high CD8 density) were found to be PD-L1 positive by CPS ($P_{trend} < 0.0001$). The overall distribution of CD8 densities and expression of PD-L1 is shown in Figure 3e.

Table 1

Relationship of PD-L1 expression by clinicopathologic factors and CD8 infiltration in small cell carcinoma of the esophagus

		PD-L1 expression in TIICs			PD-L1 expression by CPS		
Characteristics	**Total ($N=147$) [*n* (%)]**	**<1% (negative) ($n=82$, 55.8%) [*n* (%)]**	**≥1% (positive) ($n=65$, 44.2%) [*n* (%)]**	***P***	**<1 (negative) ($n=105$, 71.4%) [*n* (%)]**	**≥1 (positive) ($n=42$, 28.6%) [*n* (%)]**	***P***
Sex				0.405			0.634
Male	109 (74.1)	63 (42.9)	46 (31.3)		79 (53.7)	30 (20.4)	
Female	38 (25.9)	19 (12.9)	19 (12.9)		26 (17.7)	12 (8.2)	
Age (years)				0.276			0.498
≤60	73 (49.7)	44 (29.9)	29 (19.7)		54 (36.7)	19 (12.9)	
>60	74 (50.3)	38 (25.9)	36 (24.5)		51 (34.7)	23 (15.6)	
FFPE block age				<0.001			<0.001
>5 years	124 (84.4)	79 (53.7)	45 (30.6)		98 (66.7)	26 (17.7)	
Within 5 years	23 (15.6)	3 (2.0)	20 (13.6)		7 (4.8)	16 (10.9)	
Alcohol abuse				0.426			0.917
Yes	71 (48.3)	42 (28.6)	29 (19.7)		51 (34.7)	20 (13.6)	
No	76 (51.7)	40 (27.2)	36 (24.5)		54 (36.7)	22 (15.0)	
Tobacco abuse				0.173			0.508
Yes	97 (66.0)	58 (39.5)	39 (26.5)		71 (48.3)	26 (17.7)	
No	50 (34.0)	24 (16.3)	26 (17.7)		34 (23.1)	16 (10.9)	
Smoking index				0.518			0.330
≤400	93 (63.3)	50 (34.0)	43 (29.3)		69 (46.9)	24 (16.3)	
>400	54 (36.7)	32 (21.8)	22 (15.0)		36 (24.5)	18 (12.2)	
BMI				0.049			0.636
<25	108 (73.5)	55 (37.4)	53 (36.1)		76 (51.7)	32 (21.8)	
≥25	39 (26.5)	27 (18.4)	12 (8.2)		29 (19.7)	10 (6.8)	
Family history				0.251			0.088
Yes	32 (21.8)	15 (10.2)	17 (11.6)		19 (12.9)	13 (8.8)	
No	115 (78.2)	67 (45.6)	48 (32.7)		86 (58.5)	29 (19.7)	
Location				0.259			0.241
Upper	17 (11.6)	7 (4.8)	10 (6.8)		10 (6.8)	7 (4.8)	
Middle	111 (75.5)	62 (42.2)	49 (33.3)		79 (53.7)	32 (21.8)	
Lower	19 (12.9)	13 (8.8)	6 (4.1)		16 (10.9)	3 (2.0)	
Length (cm)				0.003			0.014
<5	89 (60.5)	41 (27.9)	48 (32.7)		57 (38.8)	32 (21.8)	
≥5	58 (39.5)	41 (27.9)	17 (11.6)		48 (32.7)	10 (6.8)	
Macroscopic tumor type				0.011			0.015
Superficial/protruding	33 (22.4)	12 (8.2)	21 (14.3)		18 (12.2)	15 (10.2)	
Medullary/mushroom/ ulcerative/intracavity	114 (77.6)	70 (47.6)	44 (29.9)		87 (59.2)	27 (18.4)	
Other histologic components				0.351			0.064
Pure small cell	116 (78.9)	67 (45.6)	49 (33.3)		87 (59.2)	29 (19.7)	
Mixed small cell	31 (21.1)	15 (10.2)	16 (10.9)		18 (12.2)	13 (8.8)	
T stage				0.028			<0.001
T1	45 (30.6)	19 (12.9)	26 (17.7)		23 (17.7)	22 (15.0)	
T2/T3/T4	102 (69.4)	63 (42.9)	39 (26.5)		82 (55.8)	20 (13.6)	
N stage				0.697			0.783
N0	50 (34.0)	29 (19.7)	21 (14.3)		35 (23.8)	15 (10.2)	
N1/N2/N3	97 (66.0)	53 (36.1)	44 (30.0)		70 (47.6)	27 (18.4)	
TNM stage				0.171			0.135
I	29 (19.7)	15 (10.2)	14 (9.5)		17 (11.6)	12 (8.2)	
II	49 (33.3)	23 (15.6)	26 (17.7)		34 (23.1)	15 (10.2)	
III	69 (46.9)	44 (30.0)	25 (17.0)		54 (36.7)	15 (10.2)	
CD8 density/mm^2				0.001			<0.001
Negative (<195.9)	104 (70.7)	67 (45.6)	37 (25.2)		83 (56.5)	21 (14.3)	
Positive (>195.9)	43 (29.3)	15 (10.2)	28 (19.0)		22 (15.0)	21 (14.3)	
Cancer-specific mortality				0.004			0.051
Yes	85 (57.8)	56 (38.1)	29 (19.7)		66 (44.9)	19 (12.9)	
No	62 (42.2)	26 (17.7)	36 (24.5)		39 (26.5)	23 (15.6)	

CPS, Combined Positive Score; FFPE, formalin-fixed paraffin-embedded; PD-L1, programmed cell death-ligand 1; TIIC, tumor-infiltrating immune cell.

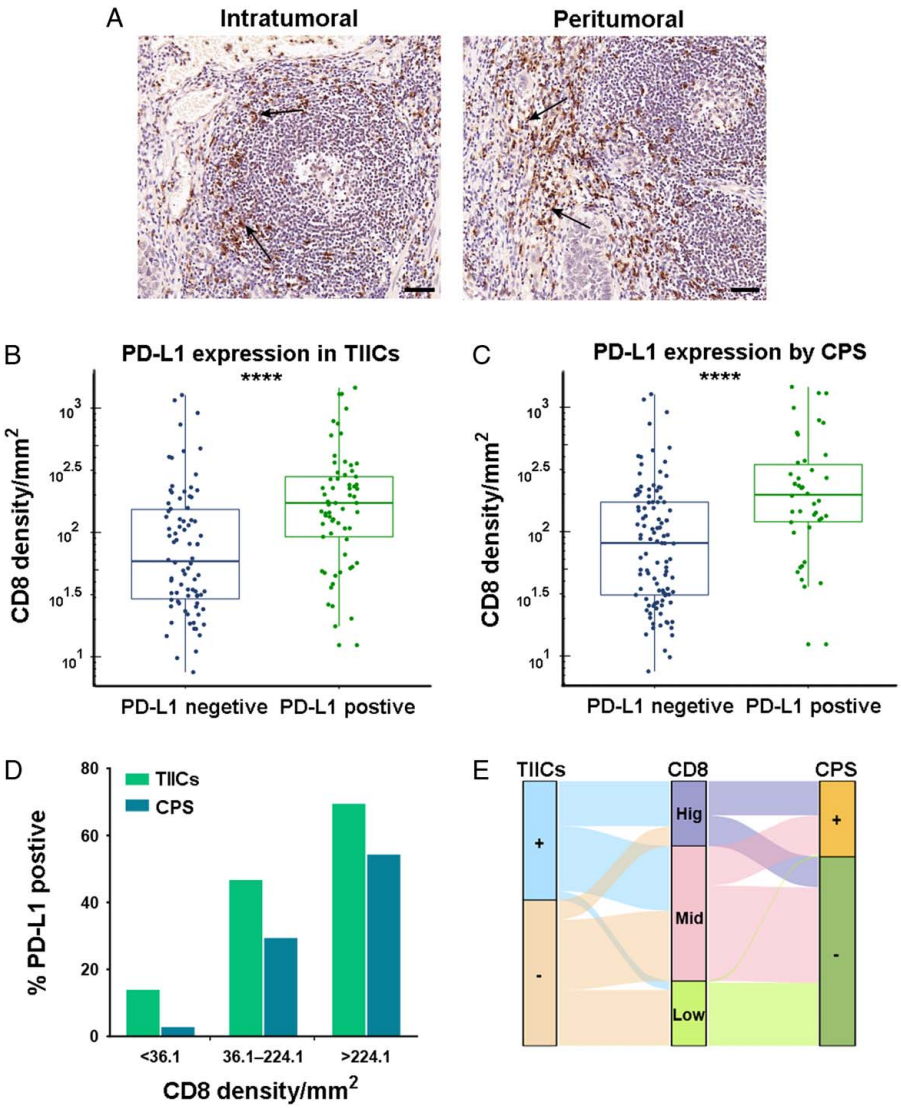

Figure 3. CD8$^+$ T-cell infiltration and the association with PD-L1 patterns in SCCE. (A) CD8$^+$ T-cell infiltration in the intratumoral compartment (left panel, ×200) and the peritumoral compartment (right panel, ×200). Scale bars, 50 μm. The distributions of CD8$^+$ T-cell density in SCCEs with or without expression of PD-L1 in TIICs (B) or by CPS (C). (D) PD-L1 expression in TIICs or by CPS increases with increasing CD8 density. (E) The landscape of distributions of CD8 densities and PD-L1 status in SCCE. ****$P < 0.0001$. CPS, Combined Positive Score; PD-L1, programmed cell death-ligand 1; SCCE, small cell carcinoma of the esophagus; TIIC, tumor-infiltrating immune cell.

Prognostic features of expression of programmed cell death-ligand 1 and CD8$^+$ T-cell presence in small cell carcinoma of the esophagus

We assessed the relationship between the expression of PD-L1 or CD8$^+$ T-cell infiltration and clinical outcomes in patients with SCCE. Patients who were found to have positive PD-L1 staining in their TIICs were significantly less likely to experience relapse [Fig. 4a, hazard ratio (HR) = 0.6129, 95% CI: 0.4203–0.8938, $P = 0.0099$] or death (Fig. 4b, HR = 0.4669, 95% CI: 0.3050–0.7147, $P = 0.0005$) relative to patients who did not express PD-L1 in their TIICs. For RFS, the *c*-index of PD-L1 in TIICs is 0.648 (0.549-0.747), time-dependent receiver operating characteristic (ROC) curve were 0.599, 0.624, and 0.628 at 1, 3, and 5 years, respectively. And for OS, the *c*-index of PD-L1 in TIICs is 0.717 (0.611–0.823), time-dependent ROC were 0.723, 0.667, and 0.657 at 1, 3, and 5 years, respectively. The CPS PD-L1-positive group experienced significantly longer OS (Fig. 4d, HR = 0.6027, 95% CI: 0.3825–0.9494, $P = 0.0453$) than the PD-L1-negative group. However, no significant difference was identified in group-specific rates of RFS (Fig. 4c, HR = 0.6711, 95% CI: 0.4481–1.005, $P = 0.0671$). We divided the CD8$^+$ T-cell densities into positive (> 195.9 cells/mm^2) and negative (< 195.9 cells/mm^2) categories. The Kaplan–Meier analysis suggested that patients with positive CD8$^+$ T-cell infiltrates had significantly longer RFS (Fig. 4e, HR = 0.4561, 95% CI: 0.3088–0.6738, $P = 0.0004$) and OS (Fig. 4f, HR = 0.4233, 95% CI: 0.2726–0.6573, $P = 0.0008$). For RFS, *c*-index of CD8$^+$ T cell is 0.690 (0.577–0.804), time-dependent ROC were 0.522, 0.604, and 0.555 at 1, 3, and 5 years, respectively. And for OS, *c*-index of CD8$^+$ T cell is 0.688 (0.556-0.820),

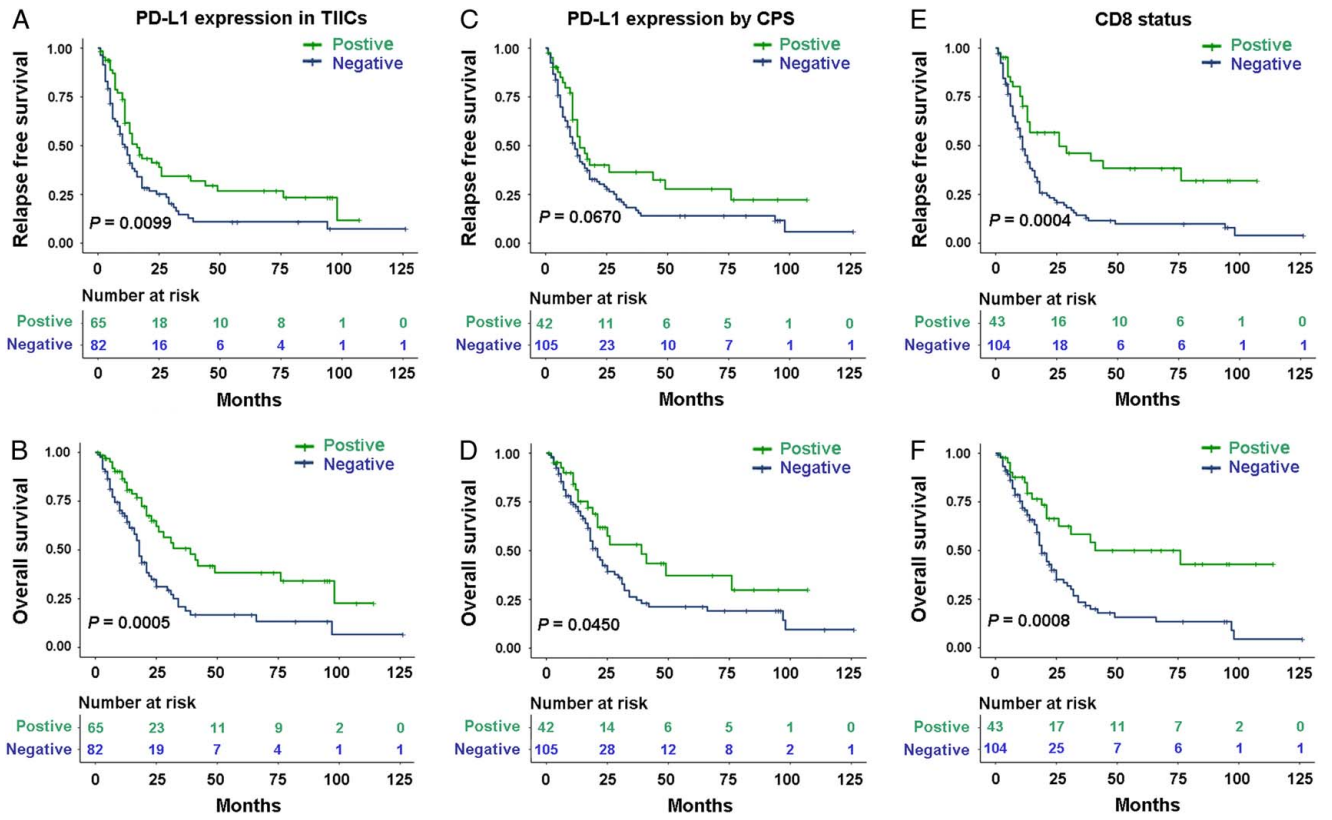

Figure 4. Correlations of PD-L1 and CD8 status and survival in small cell carcinoma of the esophagus. Relapse-free survival and overall survival in patients with small cell carcinoma of the esophagus according to PD-L1 expression in TIICs (A and B), PD-L1 expression by CPS (C and D), and CD8 status (E and F). CPS, Combined Positive Score; PD-L1, programmed cell death-ligand 1; TIIC, tumor-infiltrating immune cell.

time-dependent ROC were 0.647, 0.589, and 0.575 at 1, 3, and 5 years, respectively.

Next, we explored whether the expression of PD-L1 and $CD8^+$ T-cell density were independent significant predictors of prognosis for patients with SCCE. The univariate analysis (Supplementary Table 3, Supplemental Digital Content 4, http://links.lww.com/JS9/A5) revealed that macroscopic tumor type ($P = 0.0058$), tumor length ($P = 0.0087$), T stage ($P = 0.0036$), TNM stage ($P = 0.0108$), treatment modality ($P = 0.0027$), PD-L1 positive in TIICs ($P = 0.0006$), PD-L1 positive by CPS ($P = 0.0480$) and $CD8^+$ T-cell status ($P = 0.0012$) were significantly associated with OS. In contrast, macroscopic tumor type ($P = 0.0050$), tumor length ($P = 0.0077$), N stage ($P = 0.0439$), TNM stage ($P = 0.0085$), treatment modality ($P = 0.0164$), PD-L1 positive in TIICs ($P = 0.0115$) and $CD8^+$ T-cell status ($P = 0.0006$) were found to be significantly associated with RFS. Multivariate analysis demonstrated that treatment modality, PD-L1-positive staining in TIICs, and $CD8^+$ T-cell status were independent and significant predictors of OS (Table 2, $P < 0.05$). $CD8^+$ T-cell status was the only significant predictor of RFS (Table 2, $P < 0.05$).

Combined analysis of expression of programmed cell death-ligand 1 and $CD8^+$ T-cell counts

Cancer treatment protocols designed around the expression of PD-L1 and TIL density – especially $CD8^+$ TILs – have previously been proposed[31,33]. We classified the patients with SCCEs into four groups based on expression of PD-L1 in TIICs and density of CD8 cells, as previously described[33]: type I ($PD\text{-}L1^+$ and $CD8^+$), type II ($PD\text{-}L1^-$ and $CD8^-$), type III ($PD\text{-}L1^+$ and $CD8^-$), and type IV ($PD\text{-}L1^-$ and $CD8^+$). As shown in Figure 5a, the proportions of these 4 types were 19.0% (28/147), 45.6% (67/147), 25.2% (37/147), and 10.2% (15/147), respectively. Interestingly, we found a significant difference in both OS and RFS among these four different immunophenotypes, with *P*-values of 0.0001 and 0.0006, respectively (Fig. 5b and c).

Discussion

Given the grim prognosis and lack of efficacious treatment options for SCCEs, there is an urgent need for continued research and development of novel therapeutic strategies for this highly aggressive neuroendocrine malignancy. The recent widespread success of ICI immunotherapies for various other cancers[34] – specifically PD-L1 and PD-1 in SCLCs[12] – has generated hope that these strategies may be similarly effective in the treatment of small cell carcinomas, as well as SCCEs. Actually, PD-L1 has reported that had a significant prognosis in SCCEs, and positive expression PD-L1 was associated with more favorable survival[35]. But the study was limited in sample size, that not revealed the underlying mechanism between ICIs and SCCEs. This was the first comprehensive study of the expression patterns and prognostic impacts of expression of $CD8^+$ T-cell status, in

Table 2

Multivariable Cox regression analysis of PD-L1 expression, CD8 status, and clinicopathologic characteristics and survival in small cell carcinoma of the esophagus

		Relapse-free survival		Overall survival	
Variables		**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Sex	Male/female	1.3395 (0.7100–2.5271)	0.3668	1.7143 (0.8201–3.5835)	0.1519
Age	$>60/\leq 60$	0.9529 (0.6271–1.4480)	0.8211	1.0903 (0.6840–1.7377)	0.7164
Alcohol abuse	Yes/no	0.6650 (0.4095–1.0799)	0.0991	0.6117 (0.3558–1.0515)	0.0754
Tobacco abuse	Yes/no	1.1545 (0.6538–2.0387)	0.6204	1.1459 (0.6117–2.1466)	0.6707
Treatment modality	Surgery with adjuvant therapy/surgery alone	0.6716 (0.4321–1.0440)	0.0769	0.5413 (0.3296–0.8890)	0.0153
Location	Middle, lower/upper	0.8966 (0.5963–1.3483)	0.6001	0.6966 (0.4355–1.1143)	0.1314
Length	$\geq 5/<5$	1.1778 (0.7324–1.8938)	0.4996	1.1491 (0.6859–1.9252)	0.5975
Macroscopic tumor type	Medullary, mushroom, ulcerative and intracavity/superficial and protruding	1.5032 (0.7779–2.9050)	0.2252	1.5292 (0.7208–3.2441)	0.2684
T stage	T2, T3, T4/T1	0.6688 (0.3645–1.2274)	0.1941	0.7051 (0.3471–1.4322)	0.3338
N stage	N1, N2, N3/N0	1.4315 (0.8811–2.3256)	0.1474	1.3566 (0.7801–2.3592)	0.2800
PD-L1 in TIICs	Positive/negative	0.7795 (0.4970–1.2226)	0.2780	0.5868 (0.3479–0.9897)	0.0456
CD8 density/mm^2	Positive/negative	0.5290 (0.3182–0.8792)	0.0140	0.5418 (0.2970–0.9885)	0.0457

CI, confidence interval; HR, hazard ratio; PD-L1, programmed cell death-ligand 1; TIIC, tumor-infiltrating immune cell.

these 147 patients following SCCE resection, as well as the first largest analysis of the combination of PD-L1 and CD8$^+$ T-cell status as a predictor for prognosis in SCCE. In SCCEs, expression of PD-L1 and a high amount of CD8$^+$ T cells in the tumor were associated with improved survival. These results suggest that these two variables could be combined as biomarkers to identify patients with cancer who may be more likely to experience favorable outcomes. Patients can be classified into groups with significantly different prognoses on the basis of their expression of PD-L1 as well as CD8$^+$ T-cell status. These findings may enable risk-adapted therapeutic strategies and provide a strong foundation for future investigation of PD-L1 and PD-1-based immunotherapies for SCCE.

In this work, we used the mAb E1L3N to assess for PD-L1 expression in SCCEs. As the PD-L1 antibody 22c3 from DAKO has been widely used in clinical trials of SCLCs for PD-L1 testing[36], we first considered using 22c3 for this primary investigation SCCEs. However, in FFPE blocks that had been stored for more than 5 years, the expression of PD-L1 using 22c3 was almost undetectable (data are not shown). This finding is in line with PD-L1 testing recommendations, stating that 22c3 is not suggested for FFPE blocks older than 3 years[37]. Considering the huge spans of time (>30 years) of our samples in this research, we abandoned the use of 22c3. Among other PD-L1 antibodies, we finally selected E1L3N for testing for two reasons. First, Rimm *et al.*[38] proved that the staining performance of E1L3N was comparable with 22C3. Also, E1L3N was previously confirmed for use in FFPE blocks of pulmonary and extrapulmonary small cell carcinomas aged more than 10 years[28,39].

Herein, we measured the expression of PD-L1 in TCs as well as in TIICs in tissue samples from patients who had SCCE. Interestingly, none of the 147 cases showed PD-L1 protein expression in TCs. When we checked the results of 22c3 in FFPE samples of SCCEs within 5 years, we found the same result (data not shown). This suggests that SCCEs are analogous to SCLCs and other extrapulmonary small cell carcinomas, which rarely express PD-L1, especially in TCs[12,39,40]. However, expression of PD-L1 was found in TIICs in almost half of the SCCEs. This finding agrees with previous studies that found PD-L1 was wildly expressed in TIICs from small cell carcinomas[28,39]. Since expression of PD-L1 in TCs or TIICs has not been shown to be predictive of outcomes in patients with SCLC in ICI-based clinical trials[41,42], and CPS was confirmed as a predictive biomarker in KEYNOTE-158[29]. When we measured the contents of PD-L1 by CPS in SCCEs, we found that 28.6% of patients were stratified into the CPS-positive group. This seems lower than that reported in SCLCs in KEYNOTE-158[43], which had a positive rate of 45.7% (42/92). Considering that PD-L1 expression diminishes in FFPE samples after years of storage related to photo-oxidization or antigen degradation, we classified the samples into two categories: FFPE blocks aged more than 5 years (before 2015) and FFPE blocks aged within 5 years (2015–2019). Interestingly, the percentage of PD-L1 positive by CPS in SCCEs within 5 years was as high as 69.6% (16/23), much higher than 45.7% in SCLCs. Thus, ICI-based immunotherapies have promise as a treatment approach for SCCEs. One prior report found that the expression of PD-L1 (E1L3N) by CPS was 35% in a relatively small cohort ($N=34$) of patients with extrapulmonary small cell carcinoma[28], different from our results. However, no prior cases featured a clear esophageal origin. This might have caused the difference. We also explored the relationship between expression of PD-L1 and patients' clinicopathologic parameters. PD-L1 expression – assessed either in TIICs or by CPS – was associated with early-stage SCCEs. This finding suggested that patients with early-stage disease were more likely to benefit from ICI-based immunotherapies.

Despite mounting evidence, the number of CD8$^+$ TILs is clinically important for patients with or without therapies (especially for patients receiving immunotherapy) and various malignancies[44–47], the expression detail of CD8$^+$ TILs in SCCEs remains elusive. This was the first examination of CD8$^+$ TILs in SCCEs with a large population. Most previous studies used semiquantitative methods to describe the distribution of TILs in tumor regions[48–50]. Cancer-related lymphocytic infiltration patterns are impossible to duplicate or compare between studies. Hence, to render our data as comparable as possible, we used whole slide scanning and absolute quantification to describe all densities as 'cells/mm^2.' The average density of CD8$^+$ TILs in SCCEs was 195.9 cells/mm^2, which resembles that of SCLCs. Wang *et al.*[51] reported that the genomic landscape of SCCEs was

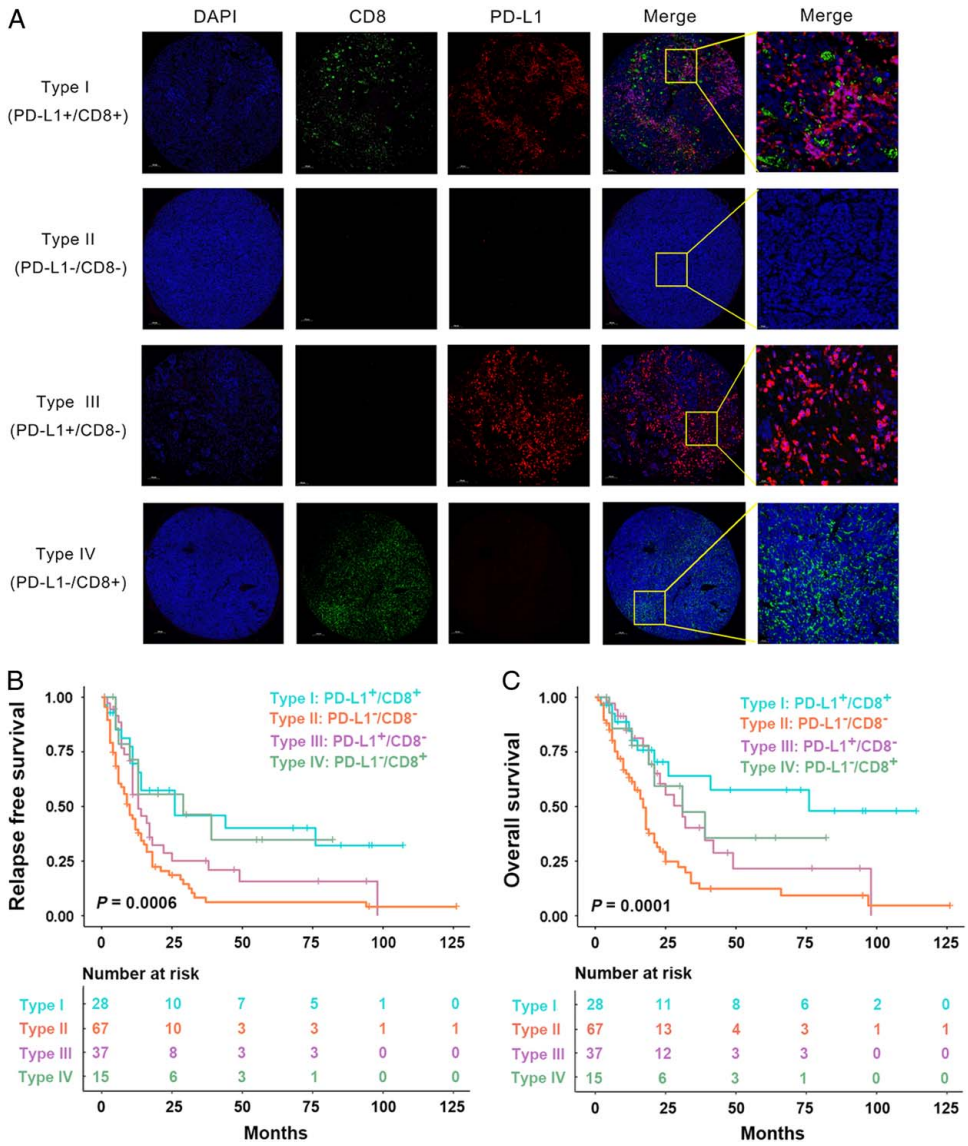

Figure 5. Survival analysis of the four different immunophenotypes in small cell carcinoma of the esophagus. (A) Representative multiple immunofluorescence images of the four different types (left panel, × 100, scale bars, 100 μm; right panel, × 400, scale bars, 20 μm). The Kaplan–Meier curves for relapse-free survival (B) and overall survival (C) in patients with small cell carcinoma of the esophagus according to programmed cell death-ligand 1 (PD-L1) expression and CD8 status.

more closely related to ESCCs. Therefore, we also compared the density of SCCEs to ESCCs and found that they were also very similar. Considering the recent successes that have stemmed from the use of immunotherapies aimed at suppressing the PD-1/PD-L1 pathway in SCLCs and ESCCs[12,52], the similarity of $CD8^+$ TILs status in these malignancies suggested that therapies based on inhibition of PD-1 or PD-L1 may also provide strong anti-tumor potential among patients with SCCEs.

We found an association between expression of PD-L1 and density of $CD8^+$ TILs with PFS and OS in 147 cases with SCCE. PD-L1 expression in TIICs or by CPS was associated with improved prognosis. Moreover, expression of PD-L1 in TIICs was an independent and significant predictor of OS. This result is in line with other pulmonary and extrapulmonary squamous cell carcinoma studies that have previously reported that expression of PD-L1 is correlated with better prognoses[53,54]. We also evaluated the prognostic significance of $CD8^+$ TILs. The $CD8^+$ TILs state is an independent predictor of both RFS and OS in SCCEs. This result is also similar to the significance of $CD8^+$ TILs in SCLCs[49]. Because SCCE is so rare, few studies have investigated its histopathologic prognostic biomarkers[55–57], and the results of these studies were restricted by their small set of samples and low statistical power. Only one previous study tested more than 80 surgically resected SCCE cancer tissue samples[25,51,55–59]. Importantly, the need for large-scale studies cannot be overemphasized because of the inherent publication bias resulting from the fact that small studies that find no difference are less likely to be written into a report or considered for publication than are small studies with significant results. Unlike previous studies, our work examined PD-L1 and $CD8^+$ TILs in the largest cohort of surgically resected SCCEs to date, and our results

confirmed that these two factors were associated with favorable clinical outcomes. Our findings support the use of PD-L1 as well as CD8$^+$ TILs as novel biomarkers to predict outcomes in patients with SCCEs.

We found more PD-L1 positive than PD-L1-negative CD8$^+$ TILs in the tumor regions of SCCEs. With CD8$^+$ TILs densities further broken into quartiles and then divided into low, mid, and high categories, 69.44% of PD-L1-positive samples in TIIC had high CD8 densities. In SCLC and melanoma[52,59], there has been a positive relationship reported between TIL infiltration and PD-L1 expression. This relationship speaks to the connection between CD8$^+$ TILs – which serve mechanistically to produce cytokines such as IFN-γ[60,61] – and the expression of PD-L1. We, therefore, propose that PD-L1 expression in SCCEs is induced by infiltrating adaptive immune cells and reflects the presence of adaptive anti-tumor immune pressure. Based on this hypothesis, our finding seems reasonable, specifically that the survival of SCCEs that stained PD-L1 positive was significantly prolonged compared with that of SCCEs that were PD-L1 negative. After categorizing patients based on the expression of PD-L1 and density of CD8$^+$ TILs, we detected the clinical significance of the four groups: type I – adaptive immune resistance (PD-L1$^+$ CD8 TILs$^+$), type II – immunologic ignorance (PD-L1$^-$ CD8 TILs$^-$), type III – intrinsic induction (PD-L1$^+$ CD8 TILs$^-$), and type IV – tolerance (PD-L1$^-$ CD8 TILs$^+$)[33]. Our results revealed that patients with different immunophenotypes displayed different prognostic features. In addition, type II patients showed significantly worse outcomes. As reported for melanoma and SCLCs[12,52], immunotherapy targeting PD-1/PD-L1 may benefit those with SCCEs who have a preexisting activated T cells in the tumor microenvironment, especially in type I patients.

Our study had several limitations. First, for this extremely rare malignant tumor, although we examined more samples than any previous SCCE-related study, this was a retrospective analysis and carries a risk of selection bias. Second, although we aimed to evaluate representative whole tissue sections, the inherent spatial heterogeneity of tumors presents the possibility that our results may not be representative of the PD-L1 and CD8$^+$ TIL status of the entire tumor. Advanced technologies, next-generation sequencing technology, for example, which have advantages of 'High-throughput' and accurate quantitative analysis should be applied in future exploration. Third, various methodologies and antibodies have been applied to assess the protein levels of PD-L1. Future studies using different antibodies to evaluate PD-L1 in SCCEs are needed.

Conclusion

This was the first large cohort study to reveal that about half of SCCEs express PD-L1 exclusively within immune cells. PD-L1 was more frequently expressed on CD68$^+$ TIICs in early-stage SCCEs and was associated with better outcomes. CD8$^+$ TIL status is a novel independent prognostic predictor for SCCEs. In addition, we identified four subgroups – defined based on the expression of PD-L1 and the presence of CD8 – possessed diverse prognostic characteristics. Importantly, we show for the first time that high infiltration with CD8$^+$ TILs in SCCEs was accompanied by high PD-L1 expression. This finding suggests the development of resistance against adaptive immune clearance as a mechanism of survival in these rare tumors. This mechanism may be manageable through treatment with anti-PDs. In SCCEs, PD-L1 and CD8$^+$ TIL status acted as predictive tissue biomarkers and may help improve risk-adapted treatment strategies. These findings support further investigation of the therapeutic value of anti-PD-1 and anti-PD-L1 antibodies for the management of patients with SCCE.

Ethical approval

The protocol of this study was approved by the Ethics Committee of the Cancer Hospital of the Chinese Academy of Medical Sciences. Due to retrospective nature of this study, the requirement for informed consent was waived. This work was conducted in compliance with the Declaration of Helsinki.

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Author contribution

N.S. and J.H. supervised the project, designed, edited, and led out the experiments of this study. C.Q.Z., G.C.Z., and Z.H.Z. conducted the experiments and data analysis. C.Q.Z. and Z.H.Z. prepared all the figures and tables. C.Q.Z. drafted the manuscript. G.C.Z., L.Y.X., Q.P.Z., P.W., L.D.W., Z.Y.Y., B.Z., F.W. T., Q.X., and S.G.G. collected clinical samples and provided material support. All the authors reviewed and approved the final manuscript.

Conflicts of interest disclosure

The authors declare that they have no financial conflict of interest with regard to the content of this report.

Research registration unique identifying number (UIN)

Not applicable.

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