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The prognostic value of TCF1+CD8+T in primary small cell carcinoma of the esophagus

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Abstract

TCF1+CD8+T cells are reported to exhibit stem-like properties with the ability to self-renew and differentiate into terminal effector T cells (TCF1-CD8+T cells) to enhance antitumor response. Previous studies indicated that TCF1+CD8+ tumor-infiltrating lymphocytes (TILs) are related to response to immunotherapy. However, their role in predicting prognosis for patients with primary small cell carcinoma of the esophagus (PSCCE) remains unclear. In this study, the expression of TCF1+CD8+T was analyzed by multiplex fluorescence immunohistochemistry in tumor tissues of 79 patients with PSCCE. High infiltration of TCF1+CD8+T cells had longer overall survival (OS) than low infiltration ($P = .009$, hazard ratio [HR] = 0.506). High TCF1+CD8/CD8 ratio (>21%) showed superior OS compared with low ratio (≤21%) ($P < .001$, HR = 0.394). In the validation set (n = 20), the prognostic value of TCF1+CD8+T cells on OS was also verified. TCF1+CD8+T cells are strong prognostic predictors.

KEYWORDS
prognosis, PSCCE, stem-like, TCF1, tumor microenvironment

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1 | INTRODUCTION

Primary small cell carcinoma of the esophagus (PSCCE) is one of the malignancies in the world and is characterized by low incidence, high aggressiveness, and poor prognosis.[1] Tumor infiltrating lymphocytes play an important role in tumor progression and prognosis.[2,3] Previous studies indicated that the abundance of CD8+T cells is associated with clinical benefits in many cancers, such as esophageal squamous cell carcinomas or adenocarcinomas and non–small cell lung cancer.[4,5] However, CD8+ tumor-infiltrating lymphocytes (TILs) represent a highly heterogeneous population, consisting of distinct subpopulations.[6] It is urgent to identify the composition of CD8+TILs and the subpopulation with antitumor immune response.

An effective immune response depends on progenitor cells capable of self-renewal and proliferation. In chronic lymphocytic choriomeningitis virus (LCMV) infection and cancer, T cells that express TCF-1 are defined as stem-like T cells.[7-9] Transcription factor T-cell factor 1 (TCF-1), encoded by TCF-7, is a critical transcription factor of T lymphocyte cell development.[10] TCF-1 silencing causes T progenitor cells to lose their self-renewing ability and means irreversible differentiation of effector T cells, as confirmed by mouse models.[11] In addition to persisting in tertiary lymphoid structures, the TCF1+ population is also observed in tumor area.[9,12] Recent studies indicated that TCF1+CD8+TILs exhibit stem-like properties with the ability to self-renew and differentiate into terminal effector T cells (TCF1-CD8+T cells) to maintain antitumor response.[13,14] Moreover, TCF1+CD8+T cells can undergo massive expansion in response to anti-PD-1 treatment.[15] The high infiltration of TCF1+CD8+TILs has been shown to be associated with prolonged progression-free survival (PFS) and overall survival (OS) in melanoma patients receiving checkpoint blockade.[16] Moreover, human leukocyte antigen (HLA) class 1 (HLA-I) downregulation is an immune escape mechanism in tumors.[17] It plays an important role in the antitumor effect of CD8+T cells. The survival association of combined HLA-I expression and stem-like CD8+T requires further exploration.

In this study, we performed a quantitative analysis of intratumoral stem-like T cells, evaluated its prognostic value, and assessed its association with HLA-I.

2 | MATERIALS AND METHODS

This retrospective study has been approved by the Ethics Review Board of Shandong Cancer Hospital and Institute and Shandong Provincial Hospital Affiliated to Shandong University and it conforms to the provisions of the Declaration of Helsinki. This study was a retrospective analysis and did not require informed consent from patients.

2.1 | Human samples

We collected tumor tissue from 79 patients diagnosed with PSCCE and treated in Shandong Cancer Hospital and Institute or Shandong Provincial Hospital. Of the total 79 patients, 39 patients had undergone surgery, and 40 patients had received chemotherapy or chemotherapy combined with radiotherapy. In addition, we enrolled 20 patients in the validation set. Information on tumor size, infiltration depth, node metastasis status, histological grade, TNM classification, and proliferative activity (Ki-67) was collected.

2.2 | Immunohistochemistry staining

Paraffin-embedded tumor tissues were cut to obtain serial sections (4 μm thick). Slides hatched at 65°C for 1 hour were deparaffinized and rehydrated in xylene solution and ethanol series. Antigen retrieval was performed in saline citrate buffer. After heating, the slides were cooled to room temperature and rinsed briefly with phosphate-buffered saline (PBS). Endogenous peroxidase activity was neutralized by peroxidase blocking for 15 minutes. Then, the slides were washed with PBS and treated with Protein Block for 15 minutes. Next, the slides were incubated with HLA-I antibody (Abcam, ab70328) overnight at 4°C. Then, cleaned slides were incubated with Novolink DAB substrate buffer and stained with hematoxylin, dehydrated, and placed on cover slips.

The expression levels of HLA-I were assessed by a general pathologist who was unaware of the patient's clinical outcomes. The percentage of staining positive cells was 0% (0), 1%-10% (1), 11%-30% (2), 31%-66% (3), 67%-80% (4), and >80% (5). Intensity score: 0, no staining; 1, weak; 2, gentle; 3, strong. The scale and intensity scores were added to get an overall score (range 0-8). The expression of HLA-I was defined as high when the total score was ≥5.

2.3 | Multiplex fluorescence immunohistochemistry

Tissue sections were stained using the OPAL 7-COLOR MANUAL immunohistochemistry (IHC) kit (Cat. NEL811001KT, Akoya). Paraffin-embedded tumor tissues were cut to obtain serial sections (4 μm thick). Slides hatched at 65°C for 1 hour were deparaffinized and rehydrated in xylene solution and ethanol series. Antigen retrieval was performed in saline citrate buffer (pH 6.0) at 95°C for 20 minutes. Then, the slides were placed in 0.1% Triton X100 PBS (PBST) and rinsed for 15 minutes. After 1 hour of blocking, rinse slides with PBST for 15 minutes. Then, slides were incubated with the CD4 antibody (Novus, NBP1-19371) for 2 hours at room temperature. After 15 minutes of flushing, slides were incubated with Opal Polymer HRP for 10 minutes. Next, after flushing, Opal fluorochrome was added to the slides and incubated for 10 minutes. The antigen retrieval operation and blocking were repeated, the slides were incubated with CD8 antibody (Abcam, ab199016) and TCF1 antibody (CST, C63D9) in sequence, and finally mounted with DAPI (Abcam, ab104135).

2.4 | Digital image acquisition and analysis

The stained slides were scanned and analyzed using the TissueFAXS slide scanning system based on a Zeiss Axio Imager Z2

epifluorescence microscope (Tissue Gnostics). Image acquisition was performed using the StrataQuest software (TissueGnostics). The software's workflow and data display for image cytometry of tissue sections, tissue, and cell cultures is similar to flow cytometry.[18] StrataQuest provides cellular data in dot plots to show parameters of cell structure, thus providing analysis of cell phenotypes and tissue cytometry.[19] The software creates scatterplots that visualize positive and negative cells through a gating function. A low magnification scan is used to determine the position of tissue on the slide. High magnification images were used for all downstream analyses. The resulting image is exported as a full-resolution TIFF file for each dye channel's grayscale. The tiled image is reconstructed by image mosaic algorithm. The software identified cells by recognizing DAPI-stained nuclei based on thresholds set for intensity.[20] Then, the staining intensity of the channels associated with each cell was measured. Positive cells are determined by a threshold set by the intensity of the detection of signal intensity (Figure 1).

HE staining was used to determine the intratumoral area (Figure 1). Five intratumoral regions were randomly selected and analyzed. The cell counts and the percentage of positive cells out of the total number of cells (% positive cells/all nucleated cells) were measured using the TissueQuest analysis platform. Time-dependent receiver-operating characteristic (ROC) curves were used to determine optimal cutoff values and corresponding sensitivities and specificities.

2.5 | Statistical analysis

All statistical analyses were performed with SPSS (IBM), GraphPad Prism 7, and R software (version 4.0.3). The *P*-value <.05 was defined as statistical significance. The correlation analysis between marker expression and clinicopathological features was performed by chi-squared test. The association between intratumoral TILs (CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio) and TNM stage was estimated by the Mann-Whitney U test and ANOVA. The OS rate was assessed by the Kaplan-Meier method, and group differences were compared by using the log-rank test. The Cox proportional hazard regression model, univariate analysis, and multivariate analysis were applied to confirm independent prognostic predictors. Multivariate analysis was performed for variables with *P*-values <.2 in univariate analysis.

3 | RESULTS

3.1 | Patient characteristics

We detected the expression of CD8+T cells, TCF1+T cells, TCF1+CD4+T cells, and TCF1+CD8+T cells in tumor tissues of 79 patients with PSCCE. The percentage of the above indicators in all the cells of tumor tissue were used to define expression levels. As far as we know, the cutoff value of the above indicators and the
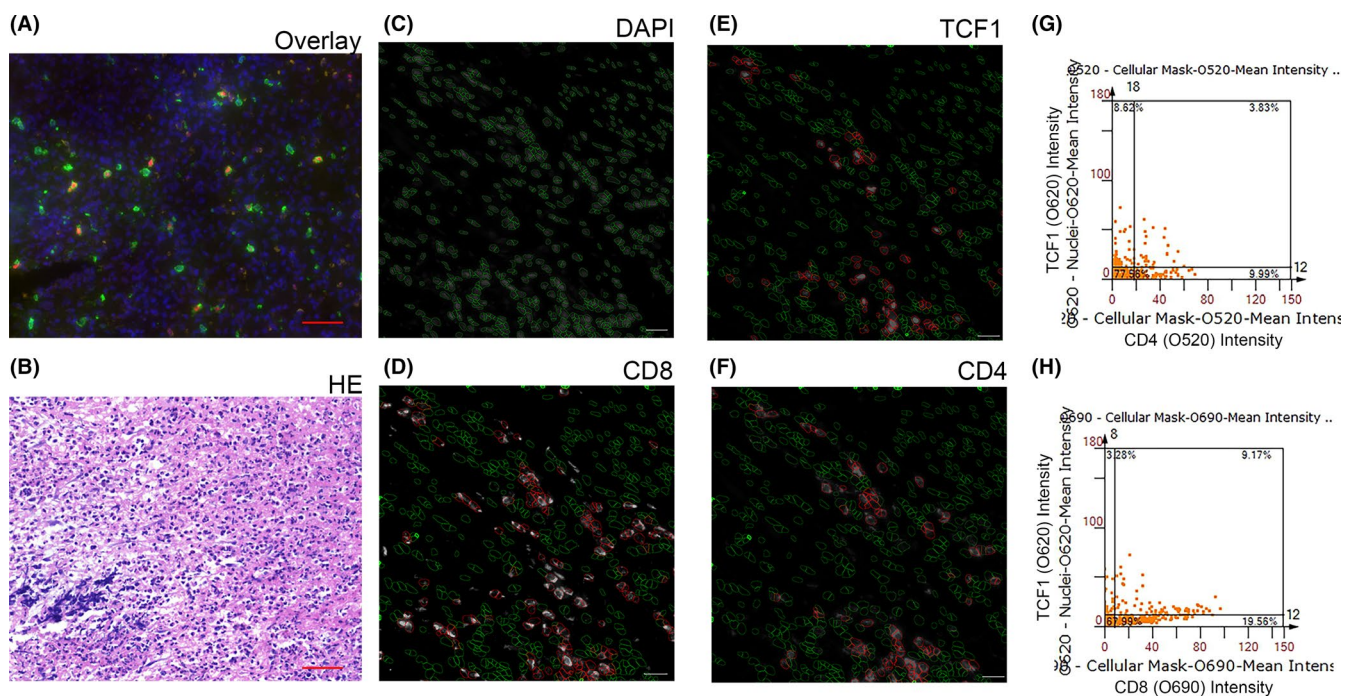
FIGURE 1 Localization and cytometric quantitation of scanned images. Nuclear segmentation (green outline) using DAPI staining identifies individual cells (C). The membrane mask (red ring) is the quantification of membrane markers for each cell (D, CD8; E, TCF1; F, CD4). Scatter plots were generated by StratQuest image analysis software based on the average staining intensity of each channel (G, H). Each dot represents one cell. The dividing line in the figure represents the intensity threshold to take into account the positive signal in each channel. Scale bars: 50 μm (A and B) and 20 μm (C, D, E, and F). CD4 (green), CD8 (yellow), TCF1 (red), and DAPI (blue)

TCF1+CD8/CD8 ratio in PSCCE are not yet clear. We used time-dependent ROC curves to determine the optimal cutoff value. The area under the curve (AUC) for CD8+T cells, TCF1+CD8+T, and TCF1+CD8/CD8 ratio was 0.633, 0.650, and 0.674, respectively (Figure 2E, F, G). The optimal cutoff of CD8+T cells, TCF1+CD8+T cells, and TCF1+CD8/CD8 ratio are 0.93% (sensitivity 49.2%, specificity 64.3%), 0.34% (sensitivity 72.3%, specificity 42.9%), and 21% (sensitivity 69.2%, specificity 89%), respectively. The AUC values for CD4+T cells, TCF1+T cells, and TCF1+CD4+T cells indicated poor discriminatory power (near 0.5). The mean values of CD8+T cells, TCF1+CD8+T cells, and TCF1+CD8/CD8 ratio are 1.30%, 0.29%, and 17%, respectively. The median values of CD8+T cells, TCF1+CD8+T cells, and TCF1+CD8/CD8 ratio are 0.97%, 0.13%, and 15%, respectively. TCF1+CD8+T had low expression in 55 (70%) patients, and HLA-I had low expression in 40 (51%) patients (Figure 2).

3.2 | Correlation between TCF1+CD8+T cells and clinicopathological characteristics

Details of the correlation are summarized in Table 1. The level of CD8+ T cells has no correlation with age, sex, alcohol, smoking history, Ki-67 level, and neuron-specific enolase (NSE) level, but it has correlation with T stage ($P = .045$), lymph node status ($P = .036$), metastatic status ($P = .001$), or TNM stage ($P = .004$). Moreover, patients with T1-T2 stage ($P = .002$), N0-N1 status ($P = .011$), or without metastasis ($P = .013$) had higher TCF1+CD8/CD8 ratio (Table 1, Figure 3). In brief, these results demonstrate that CD8+T cells and TCF1+CD8+T cells are related to tumor development, progression, and metastasis of lymph nodes.

3.3 | CD8+T cells and TCF1+CD8+T cells infiltration as prognostic biomarkers

Patients with high infiltration of CD8+T cells and TCF1+CD8+T cells have longer median OS (mOS, 25 vs 16 months, $P = .013$, hazard ratio [HR] = 0.555; 31 vs 17 months, $P = .009$, HR = 0.506; respectively; Figure 4A, B). We also proved that high TCF1+CD8/CD8 ratio showed superior OS compared with low ratio (mOS, 31 vs 16 months, $P < .001$, HR = 0.394, Figure 4C). Furthermore, we compared the impact of CD8+T and TCF1+CD8+T on the prognosis. In patients with high CD8+T infiltration, high TCF1+CD8/CD8 ratio showed better OS (mOS, 32 vs 19 months, $P = .042$, HR = 0.511 Figure 4D). We found that the OS rate decreased with
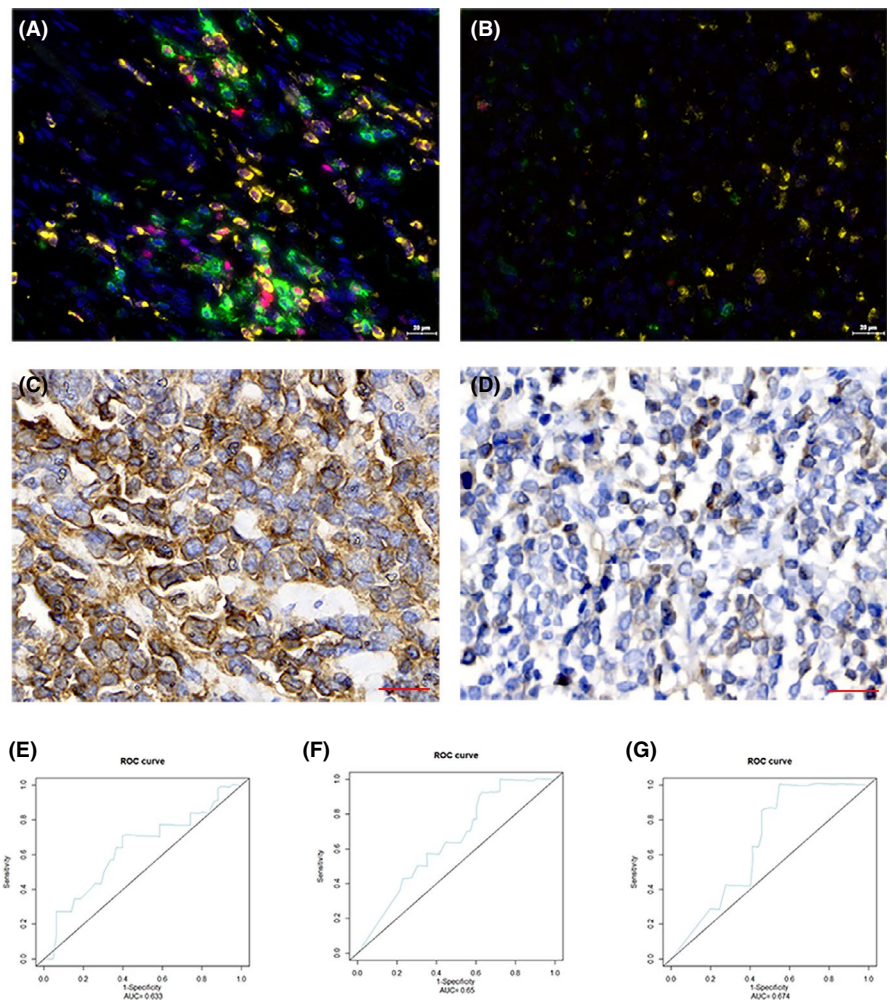
FIGURE 2 The infiltration of T cells in primary small cell carcinoma of the esophagus (PSCCE). CD4 (green), CD8 (yellow), TCF1 (red), and DAPI (blue) were stained by multiplex fluorescence immunohistochemistry. High TCF1+CD8+T cell density (A) and low TCF1+CD8+T cell density (B). High HLA-I expression (C) and low HLA-I expression (D). Receiver-operating characteristic (ROC) curves of CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio in patients with PSCCE. The area under the curve (AUC) of CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio were 0.633 (E), 0.65 (F), and 0.674 (G), respectively. Scale bars: 20 μm (A, B, F, and G)

TABLE 1 The clinicopathological characteristics of primary small cell carcinoma of the esophagus (PSCCE) Patients

Variables	CD8+T			TCF1+CD8T			TCF1+CD8/CD8		
	Low	High	P	Low	High	P	Low	High	P
Age									
<60	20	18	.320	27	11	.790	24	14	.631
≥60	17	24		28	13		28	13	
Gender									
Male	10	8	.399	15	3	.150	14	4	.212
Female	27	34		40	21		38	23	
Alcohol abuse									
NO	20	18	.320	30	8	.083	26	12	.639
YES	17	24		25	16		26	15	
T stage									
T1-T2	8	18	.045	14	12	.033	11	15	.002
T3-T4	29	24		41	12		41	12	
N stage									
N0-N1	22	34	.036	36	20	.108	32	24	.011
N2-N3	15	8		19	4		20	3	
M stage									
M0	24	40	.001	42	22	.199	38	26	.013
M1	13	2		13	2		14	1	
Ki-67									
Low	13	14	.521	19	8	.535	17	10	.993
High	14	21		22	13		22	13	

the increase of TNM staging (Figure 4E, $P < .001$), suggesting the prognostic value of TNM staging for PSCCE. We also evaluated the prognostic value of combined TCF1+CD8 and TNM stage. Although not statistically significant, in numerical terms, high TCF1+CD8 prolonged median OS in patients with stage I-II and stage III-IV (28.5 vs 35 months; 15 vs 22 months; Figure 4F, G). We verified the survival association of TCF1+CD8+T cells in the validation set. In order to validate significant results, it was also evaluated in the validation set (n = 20). Patients with high infiltration of TCF1+CD8+T cells showed longer OS (mOS, 62 vs 18 months, $P = .021$; Figure 4K).

3.4 | Effect of combined TCF1+CD8 and HLA-I expression

We also assessed the significance of TCF1+CD8+T cells expression in subgroups classified by HLA-I expression. The expression of HLA-I was not associated with OS ($P = .962$). When HLA-I expression was low, the expression of TCF1+CD8 was not related to OS (mOS, 17 vs 31 months, $P = .615$). When HLA-I was highly expressed, the expression of TCF1+CD8 was related to better OS (mOS, 16 vs 32 months, $P = .008$; Figure 4J).

3.5 | Prognostic factors in PSCCE by multivariate analysis

We analyzed the prognostic value by cox regression model, as shown in Table 2. The univariate analysis showed that T stage ($P = .006$), lymph node status ($P = .002$), metastatic status ($P < .001$), CD8+T cells ($P = .016$), TCF1+CD8+T ($P = .012$), and TCF1+CD8/CD8 ratio ($P < .001$) were correlated with OS. The multivariate analysis showed that metastatic status ($P = .002$), T stage ($P = .064$), and TCF1+CD8/CD8 ratio ($P = .004$) were correlated with OS.

These results indicated that CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio can predict the prognosis of PSCCE. Notably, the ratio of TCF1+CD8 and CD8 T cells might have a strong predictive value when compared with other markers.

4 | DISCUSSION

Central memory CD8+T cells, the earliest developmental stage of memory T cells, could provide sustained responses to maintain antitumor power efficacy.[21] TCF1 is a major regulator of the stem-like properties of central memory CD8+T cells.[13] The infiltration of TCF1+CD8+T cells in tumor tissues has been reported in melanoma,
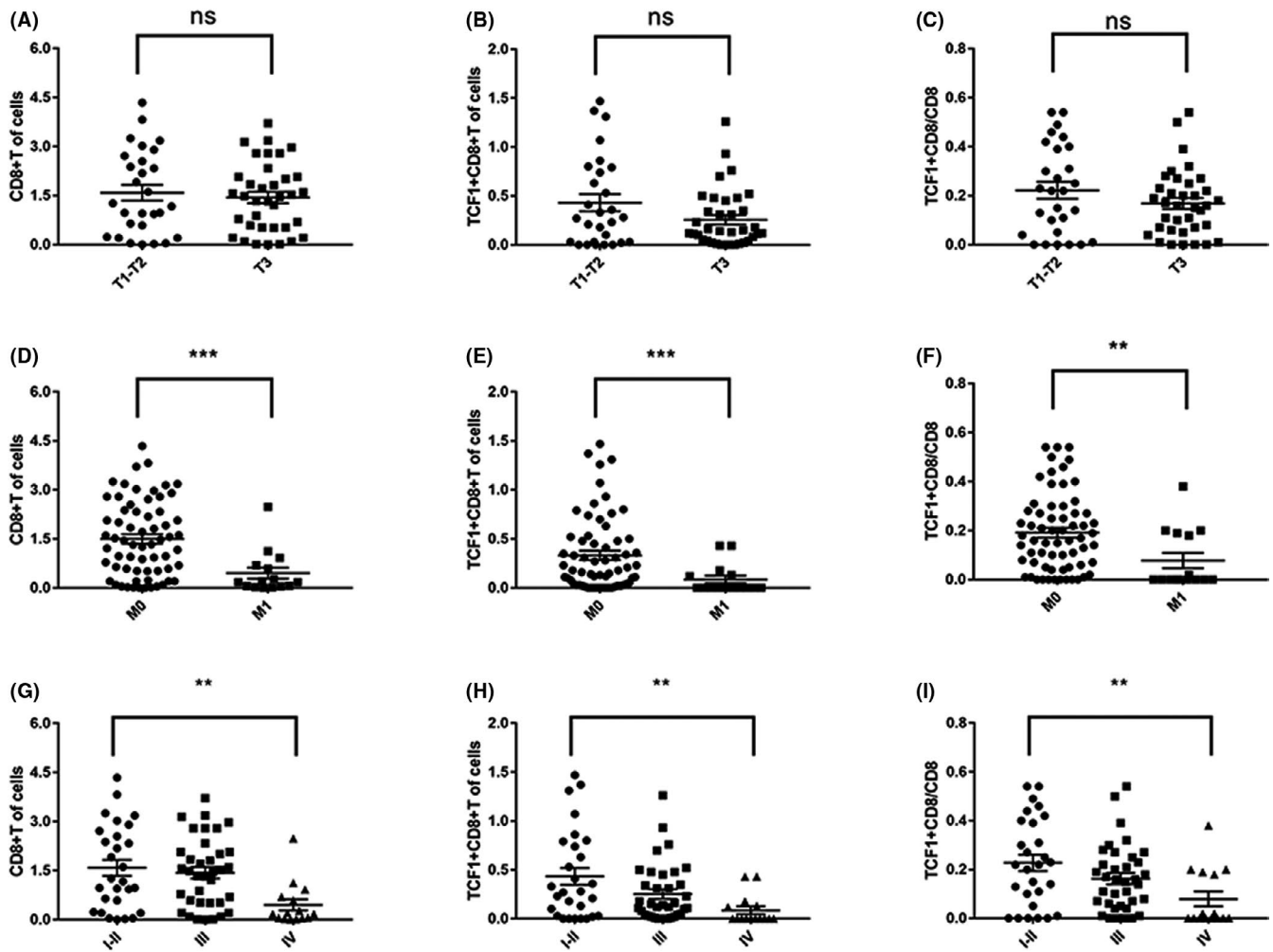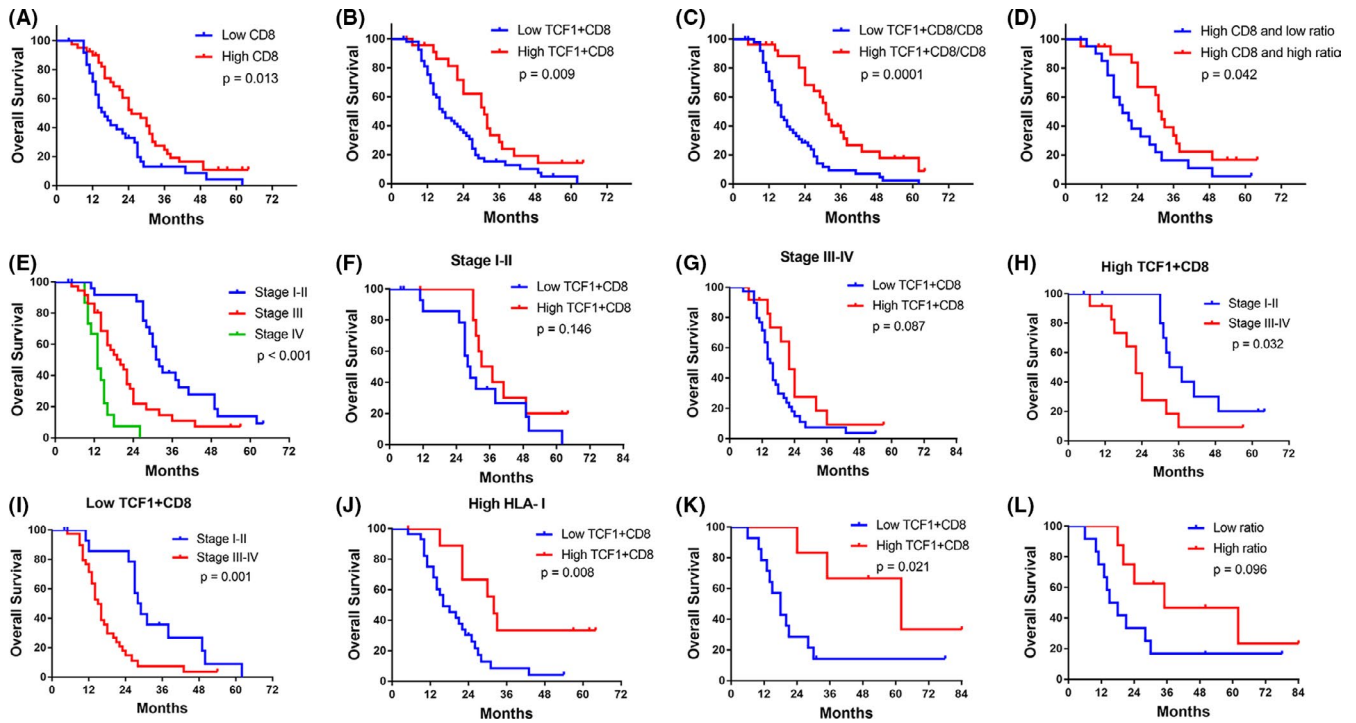
FIGURE 3 Intratumoral TILs (CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio) correlate with T stage, M stage, and TNM stage. ns, $P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$

pediatric glioma, and prostate and bladder tumors.[9,12,15] Previous studies indicated that TCF1+TIL is positively correlated with tumor regression in melanoma.[22] In this study, we performed a quantitative analysis of intratumoral TCF1+CD8+T cells by using multiplex fluorescence IHC. We proved that the infiltration of TCF1+CD8+T cells is a positive prognostic biomarker.

Stem-like T cells could stimulate persistent antitumor immune response, which is critical for adoptive T cell therapy (ACT), tumor vaccines, and immune checkpoint inhibitor (ICI) treatment. Sri Krishna et al[23] demonstrated that stem-like TILs mediate ACT response against human tumor. A preclinical study also indicated that antitumor response of tumor vaccines depends on stem-like TILs.[15] Patients with melanoma who respond to anti-PD-1 treatment have high stem-like CD8+T infiltration, and the higher the infiltration, the longer the PFS and OS .[16,22] Similarly, our results showed that high CD8+TILs and TCF1+CD8+TILs are positively related to longer OS. We also demonstrated that among patients with high CD8+TIL, patients with high TCF1+CD8/CD8 ratio were more likely to obtain better OS. High TCF1+CD8/CD8 ratio was also related to T stage, lymph node metastasis, distant metastasis, and TNM stage, which shows TCF1+CD8/

CD8 ratio might be a strong factor in tumor control and metastasis. In addition, we also discussed the prognostic value of combined TNM stage and TCF1+CD8+T because TNM stage is a powerful prognostic factor. For patients with the same TNM stage, high TCF1+CD8+T numerically prolonged OS. Besides, although CD4+T cell is involved in the activation of CD8+T cells,[24] we did not find any relationship between CD4+TIL or TCF1+CD4+TIL and survival benefits.

HLA-I is necessary for the recognition of tumor cells by CD8+T cells. The relationship between HLA-I expression and survival time has complex results. Previous studies reported that HLA-I expression was associated with better survival in pancreatic cancer, non–small cell lung cancer, and esophageal squamous cell carcinoma, while it is associated with poor prognosis in gastric cancer.[25-28] Tumor cells could evade cytotoxic CD8+T cells by downregulating HLA-I expression.[29] On the contrary, HLA-I, as an inhibitory receptor of natural killer (NK) cells, promotes tumor escape in the case of low HLA-I.[30,31] In this study, HLA-I expression was not significantly associated with survival. Patients were classified into two groups based on HLA-I expression to investigate the impact of TCF1+CD8+T cells. In the context of high HLA-I expression, patients with high TCF1+CD8+T

FIGURE 4 Kaplan-Meier survival analyses. Intratumoral TILs (CD8+T, TCF1+CD8+T, and TCF1+CD8/CD8 ratio) could predict survival benefits (A, B, C, and D). E, F, G, H, and I, Kaplan-Meier survival analysis of combined TCF1+CD8+T and TNM stage. In the case of high HLA-I expression, high TCF1+CD8+T was associated with better overall survival (OS) (J). K and L, Kaplan-Meier survival analysis for OS in the validation set

TABLE 2 Univariate analyses and multivariate analysis of prognostic markers for overall survival (OS) in primary small cell carcinoma of the esophagus (PSCCE)

	Univariate analysis		Multivariate analysis	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age				
<60 vs ≥60	0.871 (0.533-1.424)	.581		
Gender				
Male vs female	1.120 (0.838-1.496)	.444		
Alcohol abuse				
No vs yes	1.104 (0.678-1.799)	.690		
Ki-67 level				
<80 vs <80	1.321 (0.748-2.334)	.337		
NSE level				
≤16.3 vs >16.3	1.296 (0.682-2.464)	.429		
T stage				
T1-T2 vs T3-T4	2.136 (1.250-3.651)	.006	1.713 (0.970-3.028)	.064
N stage				
N1-N2 vs N3-N4	2.477 (1.410-4.351)	.002		
M stage				
M0 vs M1	4.734 (2.400-9.335)	<.001	3.062 (1.527-6.138)	.002
CD8+TILs				
Low vs high	0.545 (0.332-0.893)	.016		
TCF1+CD8+TILs				
Low vs high	0.493 (0.284-0.857)	.012		
TCF1+CD8/CD8				
Low vs high	0.366 (0.213-0.627)	<.001	0.439 (0.249-0.775)	.004

Abbreviations: CI, confidence interval; HR, hazard ratio; NS, not significant; NSE, neuron-specific enolase; OS, overall survival.

were associated with better clinical benefits. In the case of low HLA-I expression, TCF1+CD8+T was not significantly associated with prognosis. This may be related to the fact that the limited loss of HLA-I weakened the antigen recognition and thus attenuated the immune response.

Stem-like CD8+T cells have been reported to exist near the antigen-presenting cells (APCs) gathering area.[9] TCF1+PD-1+CD8+T cells are also observed in tertiary lymphoid structures or "specialized vascular niches."[12,15,32] Distribution characteristics of TCF1+CD8+T might facilitate tumor-specific CD8+T cells to populate into the tumor to maintain durable antitumor response. Therefore, analysis of the spatial distribution characteristics of TCF1+CD8+T requires further study.

We localized stem-like T cells by fluorescent multiplex immuno-histochemistry and quantified them by software based on intelligent algorithms, which greatly improves the efficiency compared with traditional IHC that requires a pathologist to score. Furthermore, it should be noted that we also have some limitations. PSCCE is characterized by low incidence and poor prognosis. Only 99 patients were enrolled in our study, and 20 of them were enrolled in the validation set.

In summary, we identified the abundance of intratumoral stem-like T cells (TCF1+CD8+T) in PSCCE. High infiltration of TCF1+CD8+T cells is related to better clinical outcomes. TCF1+CD8+T cells are strong and independent prognostic predictors.

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CONFLICT OF INTEREST
The authors have no conflict of interest.

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