# Increased coexpression of PD-L1 and TIM3/TIGIT is associated with poor overall survival of patients with esophageal squamous cell carcinoma

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## ABSTRACT

**Background** Immune checkpoint (IC) blockades (ICBs) significantly improve patients' clinical outcomes with solid tumors. Because the objective response rate of single-agent ICB is limited, it is meaningful to explore the combination of ICs for immunotherapy.

**Methods** RNA sequencing data of 95 newly diagnosed patients with esophageal squamous cell carcinoma (ESCC) from The Cancer Genome Atlas (TCGA) database were used to explore the prognostic significance of ICs. The results were validated by immunohistochemistry of 58 ESCC tissue samples from our clinical center.

**Results** The results of both TCGA and validation data suggested that high expression of programmed cell death 1 ligand 1 (PD-L1), T-cell immunoglobulin and mucin-domain-containing-3 (TIM3), and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) was associated with poor overall survival (OS) of patients with ESCC. Importantly, PD-L1/TIM3 or PD-L1/TIGIT was the optimal combination for predicting poor OS and short restricted mean survival time of patients with ESCC and was an independent prognostic factor. Moreover, a nomogram model constructed by PD-L1, TIM3, and TIGIT together with the primary tumor, regional lymph node, distant metastasis stage could provide a concise and precise prediction of 1-year and 2-year OS rates and median survival time. PD-L1/TIM3 or PD-L1/TIGIT had a positive correlation with CD8+ T cells. Notably, PD-1 and TIM3/TIGIT were primarily coexpressed on CD8+ tumor-infiltrating lymphocyte in patients with ESCC by multiplexed immunofluorescence.

**Conclusion** High expression of ICs was associated with poor OS of patients with ESCC. PD-L1/TIM3 and PD-L1/TIGIT were the optimal combinations for predicting OS, which might be potential targets for future ICBs therapy of ESCC.

## INTRODUCTION

Esophageal cancer is one of the most common gastrointestinal cancers globally.[1] [2] Esophageal squamous cell carcinoma (ESCC) accounts for approximately 90% of esophageal cancers, often at an advanced stage when initially diagnosed.[3] [4] Even with multimodality treatments (surgery, radiotherapy, chemotherapy, and targeted therapy), the prognosis of patients with ESCC remains unfavorable, with a 5-year survival≤50%.[5] Therefore, there is an urgent need to develop new treatment strategies to improve the outcome of patients with ESCC.

In recent years, immunotherapy with immune checkpoint (IC) blockades (ICBs) has shown promising effects in patients with solid tumors.[6] [7] The expression of ICs will lead to tumor cells' immune escape, promoting tumor progression.[8] ICBs can significantly improve the outcome of patients by targeting ICs, including the programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) axis.[4] [9] New ICs, such as T-cell immunoglobulin and mucin-domain-containing-3 (TIM3) and T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT), are emerging immune targets in patients with ESCC.[10] [11] For current anti-PD-1/PD-L1 antibodies, the objective response rate is limited, ranging from 6.4% to 33.3%.[4] [9] [12] This may be due to the heterogeneity of expression patterns of ICs in patients with ESCC. Therefore, it is meaningful to systematically investigate the expression patterns of ICs so that more patients with ESCC can benefit from ICBs.

In this study, we investigated the expression patterns of ICs and their impact on ESCC patients' prognosis by analyzing the RNA-seq dataset from The Cancer Genome Atlas (TCGA) database and immunohistochemistry (IHC) of tissue samples from our clinical center. Then, the correlation between ICs and CD4 + and CD8+ tumor-infiltrating lymphocytes (TILs) was explored.

## MATERIALS AND METHODS
### TCGA dataset

The level 3 RNA sequencing data of 95 newly diagnosed patients with ESCC from
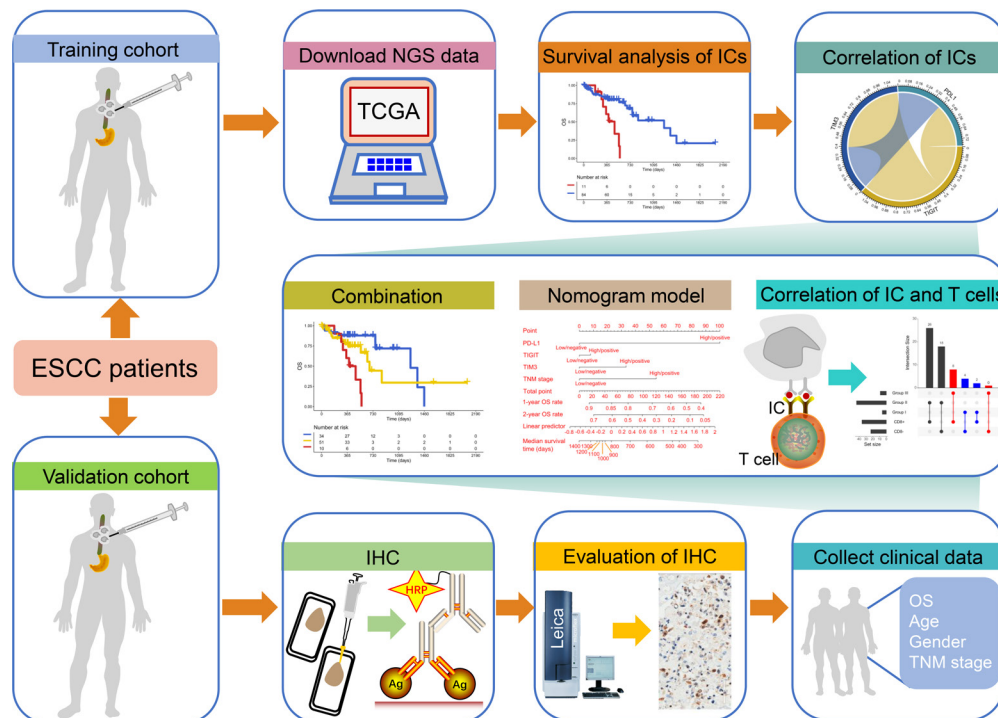
**Figure 1** Study schematics. The next-generation sequencing (NGS) data of esophageal squamous cell carcinoma (ESCC) patients from The Cancer Genome Atlas database and the immunohistochemistry (IHC) data from our clinical center were assigned as training and validation cohorts. Patients with ESCC in training and validation cohorts were not treated with immune checkpoint (IC) inhibitors. We first explored the impact of ICs on the overall survival (OS) and the correlation between ICs. Then, the effect of the combination of ICs on the prognosis of ESCC and the visualization of OS rate and survival by nomogram model were further investigated. Finally, the relationship between ICs and T cells was explored. HRP, horseradish peroxidase; TNM, primary tumor, regional lymph node, distant metastasis.

the TCGA database (https://cancergenome.nih.gov/) were acquired by the UCSC Xena platform (https://xenabrowser.net/datapages/), which was designated as a training cohort. Related clinical information, including age, gender, primary tumor, regional lymph node, distant metastasis (TNM) stage, survival time, and status, were also obtained, listed in online supplemental table S1. The ESCC dataset in the TCGA database was publicly available and therefore no approval from the local ethics committee was required.

**ESCC tissue samples**

Formalin- fixed, paraffin-embedded (FFPE) blocks were collected from 58 ESCC patients treated with pathological tumor biopsy or surgical resection at the Second Affiliated Hospital of South China University of Technology between December 1, 2012 and December 31, 2019, was assigned as a validation cohort. Patients with autoimmune diseases or other esophageal cancers (eg, adenocarcinoma) were excluded. All biopsy samples were obtained before chemotherapy or radiation therapy. According to the Union for International Cancer Control and the American Joint Committee on Cancer 8th staging system, tumors were staged. The last follow-up was conducted on February 21, 2021, and the median follow-up time was 397 days (range: 18–1517 days). The clinical information is shown in online supplemental table S1. The key inclusion criteria in training and validation cohorts were

as follows: (1) Newly diagnosed patients with cytopathological confirmed ESCC and (2) aged 18 years or older. Major exclusion criteria were as follows: (1) Prior treatments of chemotherapy, targeted therapy, radiotherapy, or surgery and (2) no complete follow-up information.

**Immunohistochemistry**

Tissue slides (4 μm thickness) were prepared from FFPE samples, deparaffinized, and rehydrated through graded ethanol. Then, the slides were boiled in ethylenediaminetetraacetic (EDTA) (1 mM, pH 8.0) buffer in a microwave oven for antigen retrieval. The endogenous peroxidase activity was quenched by using 0.3% $H_2O_2$ and the non-specific binding was blocked by incubating in 5% goat serum. After this, the slides were incubated with the primary antibodies: rabbit anti-human PD-L1 (Abcam, ab205921, 1:200), rabbit anti-human TIGIT (Abcam, ab243903, 1:200), rabbit anti-human TIM3 (Abcam, ab185703, 1:400), and rabbit anti-human CD8 (Abcam, ab4055, 1:200). Then, the slides were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO EnVision Detection Kit) and visualized using the diaminobenzidine solution in the DAKO Kit. After hematoxylin counterstaining, the slides were dehydrated and sealed. The slides with immunoreactivity were scored by five randomly and averagely selected high-power fields. The scoring rules were as follows: 0, negative/trace level of staining (<5% cells are positive);
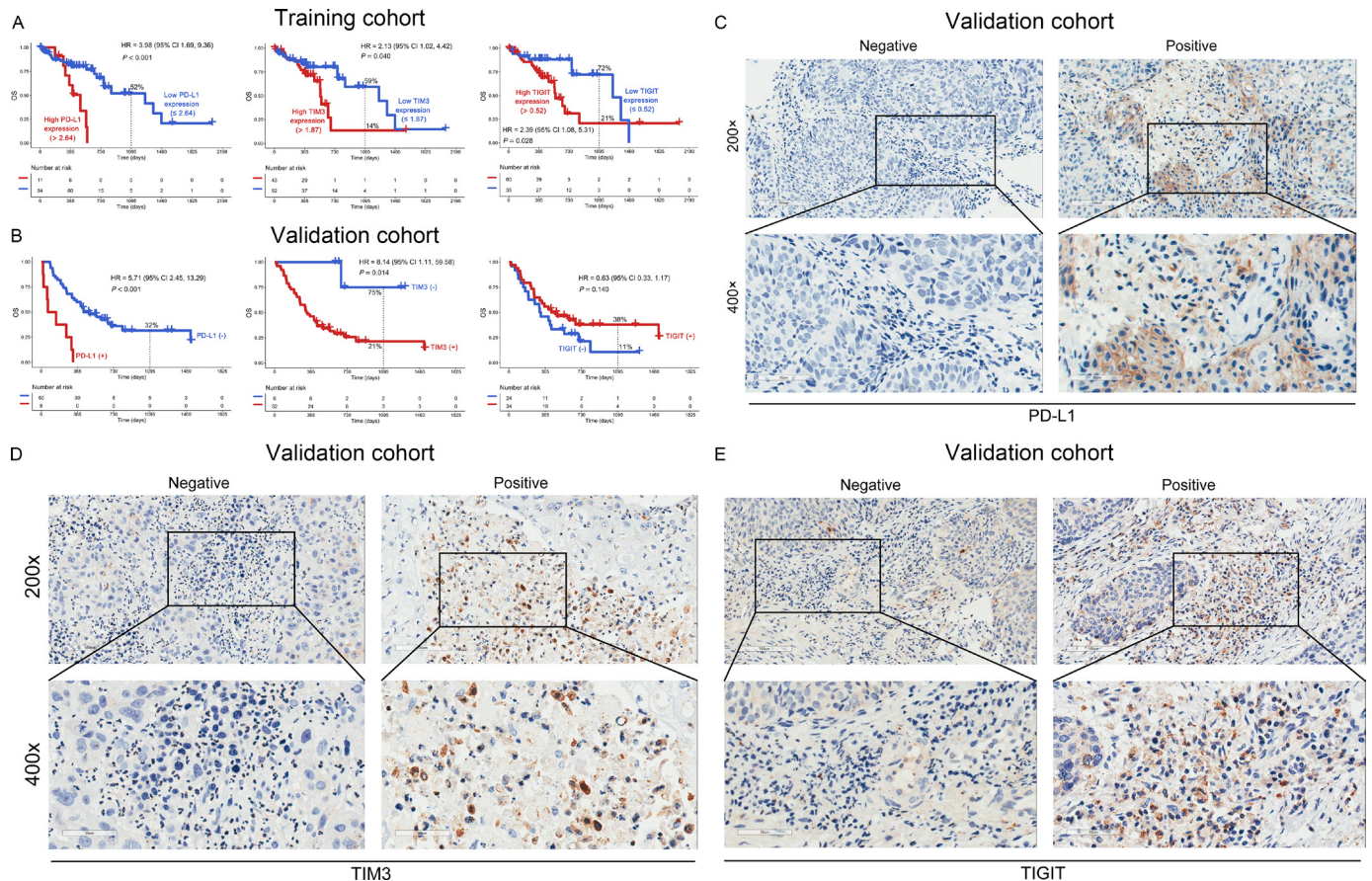
**Figure 2** OS analysis of ICs in the training and validation cohorts. The mRNA and protein expression levels of PD-L1 (left panel), TIM3 (middle panel), and TIGIT (right panel) had an impact on the OS rate of patients with ESCC in the training (A) and validation (B) cohorts. Representative micrographs of PD-L1 (C), TIM3 (D), and TIGIT (E) protein expression within the tumor tissue. ESCC, esophageal squamous cell carcinoma; ICs, immune checkpoints; OS, overall survival; PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3.

1+, weak staining (>5% cells but <25% are positive but stained with weak intensity); 2+, moderate staining (>25% cells but <50% are positive and stained with moderate intensity); and 3+, intense staining (>50% cells are positive and stained with vigorous intensity), respectively. The H-score was the multiplication of staining intensity and staining range. The cells with immunoreactivity scores of 0 were classified as negative; other scores were positive. PD-L1 analysis was performed using the combined positive score, that was, the number of PD-L1 staining cells (tumor cells and infiltrating leucocytes) divided by the total number of viable tumor cells, then multiplied by 100. For the other immunohistochemical markers, positive cells were evaluated by infiltrating leucocytes.

## Multiplexed immunofluorescence

For immunofluorescence staining, the slides are deparaffinized and rehydrated with graded ethanol and the process of antigen retrieval and non-specific binding blocking is the same as the previous IHC.

For the multiplexed immunofluorescence of CD4, CD8, PD-1, TIM3, TIGIT, and DAPI (for nuclear staining) in the FFPE tissue, we used a validated multiple fluorescence

staining kit from Servicebio (Wuhan, China), according to the manufacturer's recommendation. The following primary antibodies were used: mouse anti-human PD-1 (Abcam, ab52587, 1:200), rabbit anti-human TIGIT (Abcam, ab243903, 1:200), rabbit anti-human TIM3 (Abcam, ab185703, 1:400), and rabbit anti-human CD8 (Abcam, ab4055, 1:200). After the slides are incubated with each primary antibody and the corresponding HRP-labeled secondary antibody, the slides need to be labeled with different fluorescent dyes. The sample scanning, spectral unmixing, and quantification of signals were conducted with the panoramic MIDI Pathology Imaging System, using the CaseViewer V.2.3 software of 3DHISTECH.

## Construction of nomogram model

For the prognostic model in this study, we referred to the reporting recommendations for tumor marker prognostic studies and our previous report on constructing a nomogram model for predicting the prognosis of patients with cancer.[13–16] The nomogram model for predicting overall survival (OS) rate and median survival time of patients with ESCC was constructed by 'foreign'
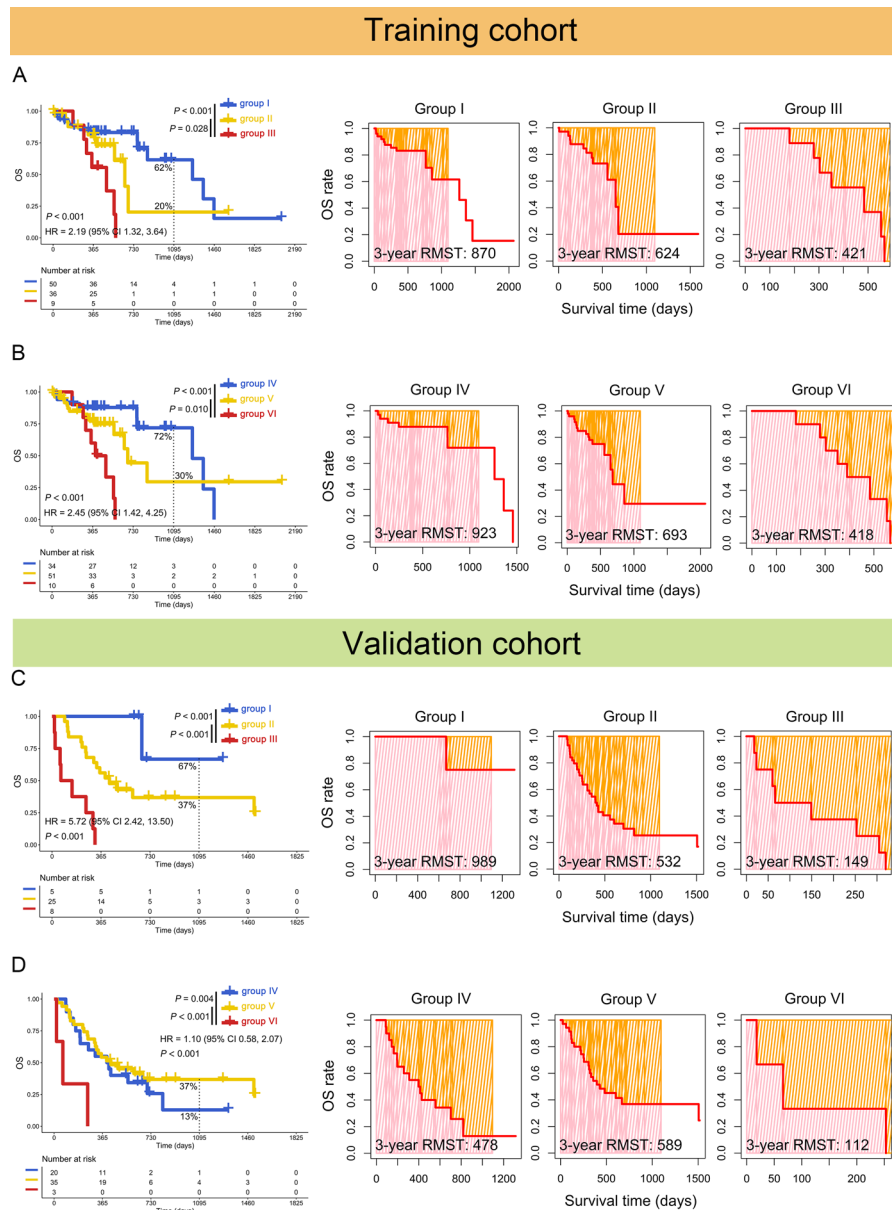
**Figure 3** Increased coexpression of PD-L1/TIM3 or PD-L1/TIGIT predicted poor OS of patients with ESCC. Kaplan-Meier (left panel) and restricted mean survival time (RMST) (right panel) curves were plotted based on the different groups of mRNA PD-L1/TIM3 (A) or PD-L1/TIGIT (B) in the training cohorts. The results of Kaplan-Meier (left panel) and RMST (right panel) curves of protein PD-L1/TIM3 (C) or PD-L1/TIGIT (D) were confirmed in the validation cohorts. Group I: PD-L1$^{lo}$TIM3$^{lo}$ or PD-L1 (−)/TIM3 (−); group II: PD-L1$^{hi}$TIM3$^{lo}$ or PD-L1$^{lo}$TIM3$^{hi}$, PD-L1 (+)/TIM3 (−) or PD-L1 (−)/TIM3 (+); group III: PD-L1$^{hi}$TIM3$^{hi}$ or PD-L1 (+)/TIM3 (+); group IV: PD-L1$^{lo}$TIGIT$^{lo}$ or PD-L1 (−)/TIGIT (−); group V: PD-L1$^{hi}$TIGIT$^{lo}$ or PD-L1$^{lo}$TIGIT$^{hi}$, PD-L1 (+)/TIGIT (−) or PD-L1 (−)/TIGIT (+); and group VI: PD-L1$^{hi}$TIGIT$^{hi}$ or PD-L1 (+)/TIGIT (+). ESCC, esophageal squamous cell carcinoma; OS, overall survival; PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3.

and 'rms' packages in R (V.4.0.2, https://www.r-project.org/).[13 14] The internal and external calibration curves, through 1000 sample bootstrap, were applied to validate the nomogram model's performance in evaluating prognosis. The evaluation criteria for the nomogram's prediction performance were as follows: the predicted value of the calibration curve was around the ideal line. The area under the curve (AUC) of the time-dependent receiver operating characteristic (ROC) curve was higher than 0.5.

**Statistical analysis**

Statistical analysis was conducted by R language (V.4.0.2, https://www.r-project.org/) and SPSS software (V.22.0, IBM, Armonk, New York, USA), as appropriate. The 'surv_cutpoint' function in the 'survminer' package was used to determine the optimal cut-off values of ICs (online supplemental figure S1)[17] and a log-rank test was used to compare differences between Kaplan-Meier curves. The Spearman correlation coefficient expressed

**Table 1** Univariate and multivariate regression analysis of mRNA PD-L1/TIM3 and PD-L1/TIGIT in the training cohort

| | Univariate regression | | Multivariate regression | | | |
| | | | PD-L1/TIM3 | | PD-L1/TIGIT | |
| Variables | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
|---|---|---|---|---|---|---|
| PD-L1/TIM3 | | | | | | |
| Group I | reference | | reference | | | |
| Group II | 1.79 (0.78 to 4.11) | 0.166 | 1.42 (0.57 to 3.51) | 0.450 | | |
| Group III | 5.09 (1.89 to 13.67) | **0.001** | 4.40 (1.55 to 12.51) | **0.005** | | |
| PD-L1/TIGIT | | | | | | |
| Group IV | reference | | | | reference | |
| Group V | 1.86 (0.79 to 4.36) | 0.155 | | | 1.43 (0.57 to 3.60) | 0.449 |
| Group VI | 6.16 (2.18 to 17.37) | **0.001** | | | 4.78 (1.63 to 13.96) | **0.004** |
| Age, years | 1.04 (1.00 to 1.07) | 0.052 | | | | |
| Gender | | | | | | |
| Female | reference | | reference | | reference | |
| Male | 5.27 (1.22 to 22.71) | 0.026 | 3.03 (0.65 to 14. 02) | 0.157 | 3.49 (0.77 to 15.91) | 0.106 |
| TNM stage | | | | | | |
| Low (I, II) | reference | | reference | | reference | |
| High (III, IV) | 2.39 (1.17 to 4.90) | 0.017 | 1.75 (0.81 to 3.77) | 0.155 | 1.59 (0.72 to 3.50) | 0.248 |

Group I, PD-L1$^{lo}$TIM3$^{lo}$; group II, PD-L1$^{hi}$TIM3$^{lo}$ or PD-L1$^{lo}$TIM3$^{hi}$; group III, PD-L1$^{hi}$TIM3$^{hi}$; group IV, PD-L1$^{lo}$TIGIT$^{lo}$; group V, PD-L1$^{hi}$TIGIT$^{lo}$ or PD-L1$^{lo}$TIGIT$^{hi}$; and group VI, PD-L1$^{hi}$TIGIT$^{hi}$.
The bold values indicate that p values <0.05 in PD-L1/TIM3 and PD-L1/TIGIT are statistically significant.
CI, confidence interval; HR, hazard ratio; PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3; TNM, primary tumor, regional lymph node, distant metastasis.

the relationship between the two ICs. The correlation between the two sets of qualitative data was shown by Cramer's V.[18] The qualitative data were compared by $\chi^2$ and Fisher test, as appropriate. Comparison of quantitative data between two and multiple groups was performed

**Table 2** Univariate and multivariate regression analysis of protein PD-L1/TIM3 and PD-L1/TIGIT in the validation cohort

| | Univariate regression | | Multivariate regression | | | |
| | | | PD-L1/TIM3 | | PD-L1/TIGIT | |
| Variables | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
|---|---|---|---|---|---|---|
| PD-L1/TIM3 | | | | | | |
| Group I | reference | | reference | | | |
| Group II | 7.16 (0.97 to 52.75) | **0.053** | 8.56 (1.14 to 64.30) | **0.037** | | |
| Group III | 35.81 (4.27 to 300.18) | **0.001** | 47.11 (5.34 to 415.96) | **0.001** | | |
| PD-L1/TIGIT | | | | | | |
| Group IV | reference | | | | reference | |
| Group V | 0.74 (0.38 to 1.44) | 0.380 | | | 0.59 (0.29 to 1.20) | 0.143 |
| Group VI | 5.74 (1.58 to 20.90) | **0.008** | | | 25.18 (3.34 to 189.74) | **0.002** |
| Age, years | 0.60(0.32 to 1.12) | 0.107 | | | | |
| Gender | | | | | | |
| Female | reference | | | | | |
| Male | 1.59 (0.49 to 5.22) | 0.441 | | | | |
| TNM stage | | | | | | |
| Low (I, II) | reference | | reference | | reference | |
| High (III, IV) | 2.26 (1.02 to 5.03) | 0.046 | 2.36 (1.02 to 5.44) | 0.044 | 2.26 (1.00 to 5.10) | 0.049 |

Group I, PD-L1 (−)/TIM3 (−); group II, PD-L1 (+)/TIM3 (−) or PD-L1 (−)/TIM3 (+); group III, PD-L1 (+)/TIM3 (+); group IV, PD-L1 (−)/TIGIT (−); group V, PD-L1 (+)/TIGIT (−) or PD-L1 (−)/TIGIT (+); and group VI, PD-L1 (+)/TIGIT (+).
The bold values indicate that p values <0.1 in PD-L1/TIM3 and PD-L1/TIGIT are statistically significant.
PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3; TNM, primary tumor, regional lymph node, distant metastasis.
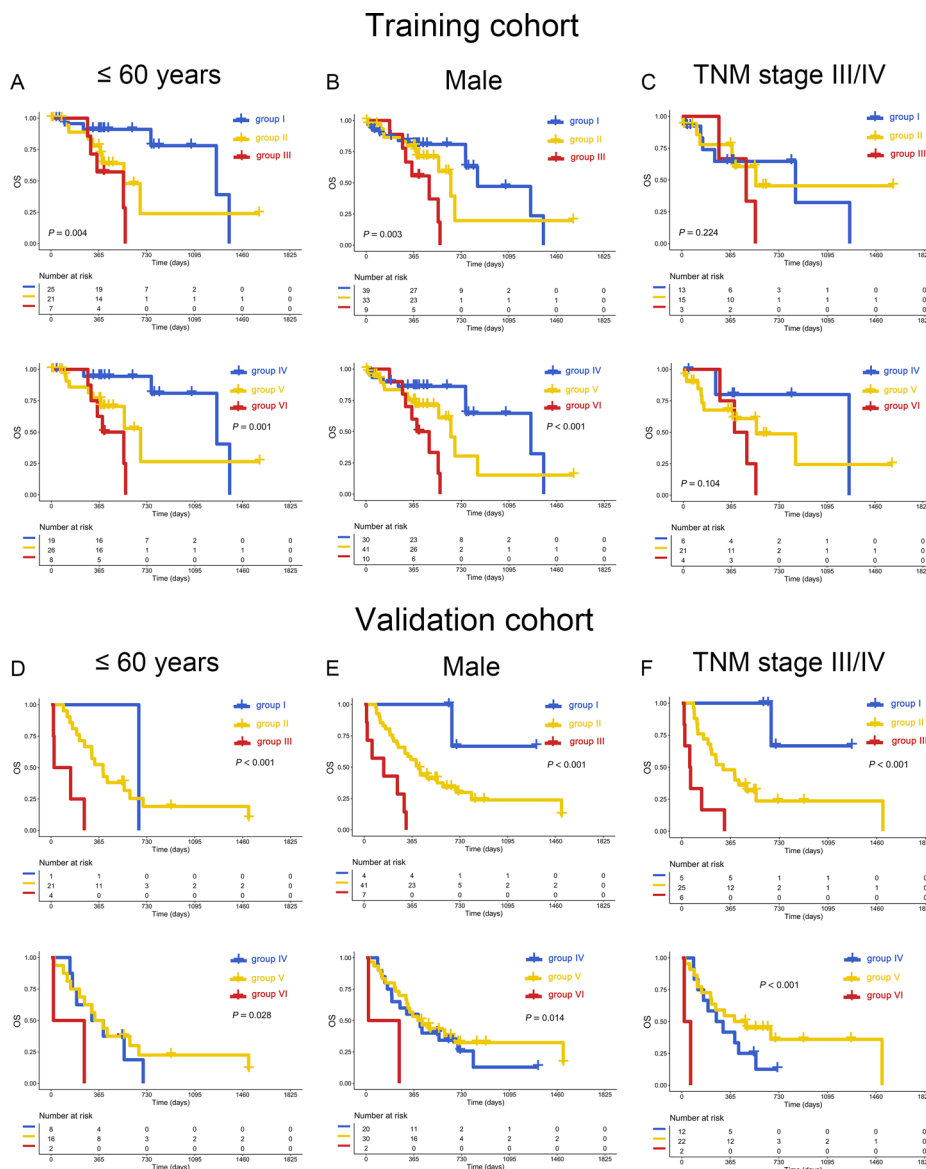
**Figure 4** Subgroup analysis of PD-L1/TIM3 and PD-L1/TIGIT. Coexpression of mRNA PD-L1/TIM3 (upper panel) or PD-L1/TIGIT (bottom panel) was associated with poor OS of patients with ESCC younger than 60 years (A), males (B), or TNM stage III/IV (C) in the training cohort. The impact of protein PD-L1/TIM3 (upper panel) or PD-L1/TIGIT (bottom panel) on OS of patients with ESCC younger than 60 years (D), males (E), or TNM stage III/IV (F) in the validation cohort. ESCC, esophageal squamous cell carcinoma; OS, overall survival; PD-L1, programmed cell death 1 ligand 1; TIGIT; T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3; TNM, primary tumor, regional lymph node, distant metastasis.

by Mann-Whitney-Wilcoxon test and Kruskal-Wallis test, respectively. A two-tailed p value<0.05 was defined as statistically significant.

## RESULTS
### OS analysis of ICs in patients with ESCC
A total of 95 newly diagnosed patients with ESCC from the TCGA database were analyzed (figure 1). High mRNA expression of PD-L1 (0% vs 52%, p<0.001), TIM3 (14% vs 59%, p=0.040), and TIGIT (21% vs 72%, p=0.028) was associated with poor 3-year OS (figure 2A). However, the mRNA expression levels of PD-1, PD-L2, cytotoxic T-lymphocyte associated protein 4 (CTLA4), lymphocyte activation gene-3, and B and T lymphocyte associated were not significantly correlated with OS (p>0.05, online supplemental figure S2A–E). Notably, the expression level of mRNA does not always match the protein; therefore, we analyzed the prognostic importance of mRNA expression and protein expression in patients with ESCC. Then, we performed IHC to detect the protein expression in the validation cohort (figure 2C–E). Poor 3-year OS was observed in patients with PD-L1 (+) or TIM3 (+) (0% vs 32%, p<0.001 and 21% vs 75%, p=0.014, respectively), but not in those with TIGIT (+) (p=0.140, figure 2B).

A



B

## Training cohort
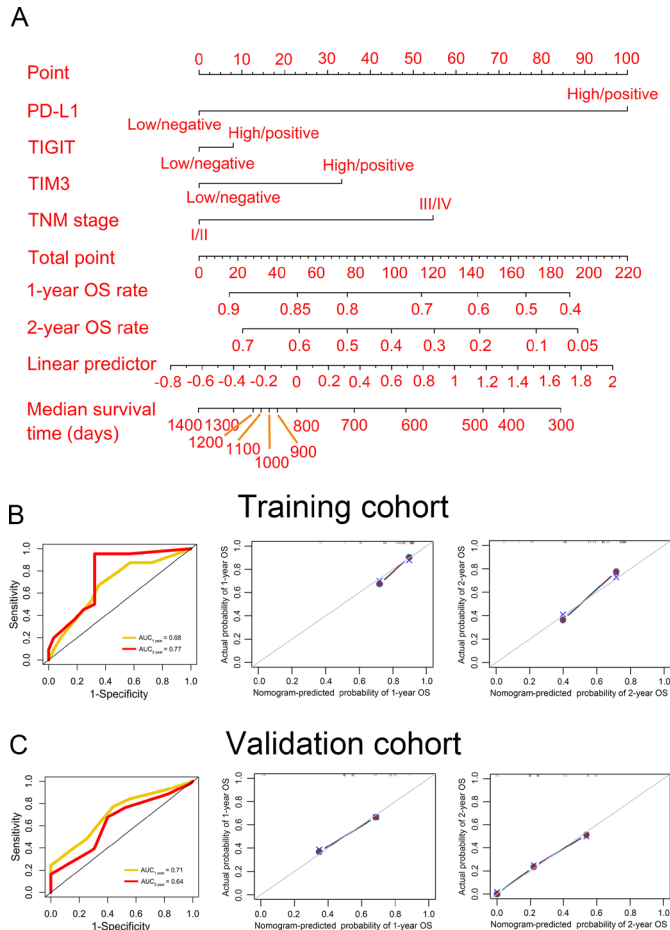


C

## Validation cohort



**Figure 5** Establishment of nomogram model. (A) A nomogram model was constructed to predict the 1-year and 2-year OS rate and median survival time of patients with ESCC. The time-dependent receiver operating characteristic (left panel) and calibration (middle and right panels) curves were used to evaluate the performance of the nomogram model in the training (B) and validation (C) cohorts. AUC, area under the curve; OS, overall survival; PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3; TNM, primary tumor, regional lymph node, distant metastasis.

### Increased coexpression of ICs was associated with poor OS

To further investigate the coexpression relationship between ICs, we performed Spearman correlation analysis. In the training cohort, there was a significant positive correlation between them (PD-L1/TIM3, R=0.39; PD-L1/TIGIT, R=0.39; TIM3/TIGIT, R=0.72; p<0.001; online supplemental figure S3A). We analyzed the coexpression pattern and found that the paired combination of mRNA PD-L1, TIM3, and TIGIT was significantly associated with poor OS (PD-L1/TIM3, p<0.001; PD-L1/TIGIT, p<0.001; TIM3/TIGIT, p=0.015; figure 3A,B and online supplemental figure S3B).

Then, the optimal ICs combination was further investigated. The concomitant high mRNA expressions of PD-L1/TIM3 (0% vs 20%, 421 vs 624 days, p=0.028) or PD-L1/TIGIT (0% vs 30%, 418 vs 693 days, p=0.01) was

associated with poor 3-year OS rate and restricted mean survival time (RMST), compared with high mRNA expression of single marker (figure 3A,B). The combination of TIGIT/TIM3 had no such effects (13% vs 35%, p=0.430, online supplemental figure S3B).

These results were confirmed in the validation cohort. The positive protein expression of dual PD-L1/TIM3 (0% vs 37%, 149 vs 532 days, p<0.001) or PD-L1/TIGIT (0% vs 37%, 112 vs 589 days, p<0.001) was related to worse 3-year OS rate and RMST (figure 3C,D) than single one. Both mRNA PD-L1/TIM3 (HR=4.40, 95% CI: 1.55 to 12.51, p=0.005) and PD-L1/TIGIT (HR=4.78, 95% CI: 1.63 to 13.96, p=0.004) were found to be independent prognostic factors (table 1). These results were again confirmed in the validation cohort (PD-L1/TIM3: HR=47.11, 95% CI: 5.34 to 415.96, p=0.001; PD-L1/TIGIT: HR=25.18, 95% CI: 3.34 to 189.74, p=0.002, table 2).

Subgroup analysis suggested that the unfavorable influence of high expression of PD-L1/TIM3 was restricted in young (≤60 y) and male patients (p=0.004 and 0.003 in the training cohort, p<0.001 in the validation cohort). Similar results can be found in PD-L1/TIGIT (p=0.001 and p<0.001 in training cohort, p=0.028 and 0.014 in validation cohort, figure 4A,B,D,E). However, in patients either old (>60 y) or female, this influence was not significant (p>0.05, online supplemental figure S4 A,B,D, E).

### Construction of a nomogram model

We first performed univariate and multivariate analysis for prognostic factors. We found that in addition to PD-L1/TIM3 and PD-L1/TIGIT, the TNM stage was also an independent predictor in the validation cohort (HR >1, p<0.05, table 2). Thereafter, PD-L1, TIM3, TIGIT, and TNM stages were used to construct a nomogram model for predicting OS (figure 5A). The point of each variable, total points, and the corresponding survival rates were listed in online supplemental table S2. The time-dependent ROC curves in both the training and validation cohorts were used to evaluate the nomogram model, indicating that the nomogram model had a good performance (AUC >0.6, figure 5B,C, left panel). Furthermore, the internal and external calibration curves confirmed that the predicted OS was around the ideal line (figure 5B,C, middle and right panel).

### Relationship between PD-L1/TIM3 or PD-L1/TIGIT coexpression and the infiltration of CD4+/CD8+ TILs in ESCC

Regarding the complex interactions among the immune microenvironment, we analyzed the correlation between PD-L1, TIM3, TIGIT, and CD4 + or CD8+ TILs (figure 1). PD-L1/TIM3 and PD-L1/TIGIT were positively correlated with CD8+TIL in the training cohort (figure 6A). However, although the ratio of CD4 +TIL was different among different PD-L1/TIM3 groups, this ratio in the PD-L1hiTIM3hi group was not higher than those in the PD-L1hi or TIM3hi group (ratio: 0.14 vs 0.15 vs 0.12, p=0.057, online supplemental figure S5A). Similar finding was also observed for PD-L1/TIGIT (ratio: 0.14
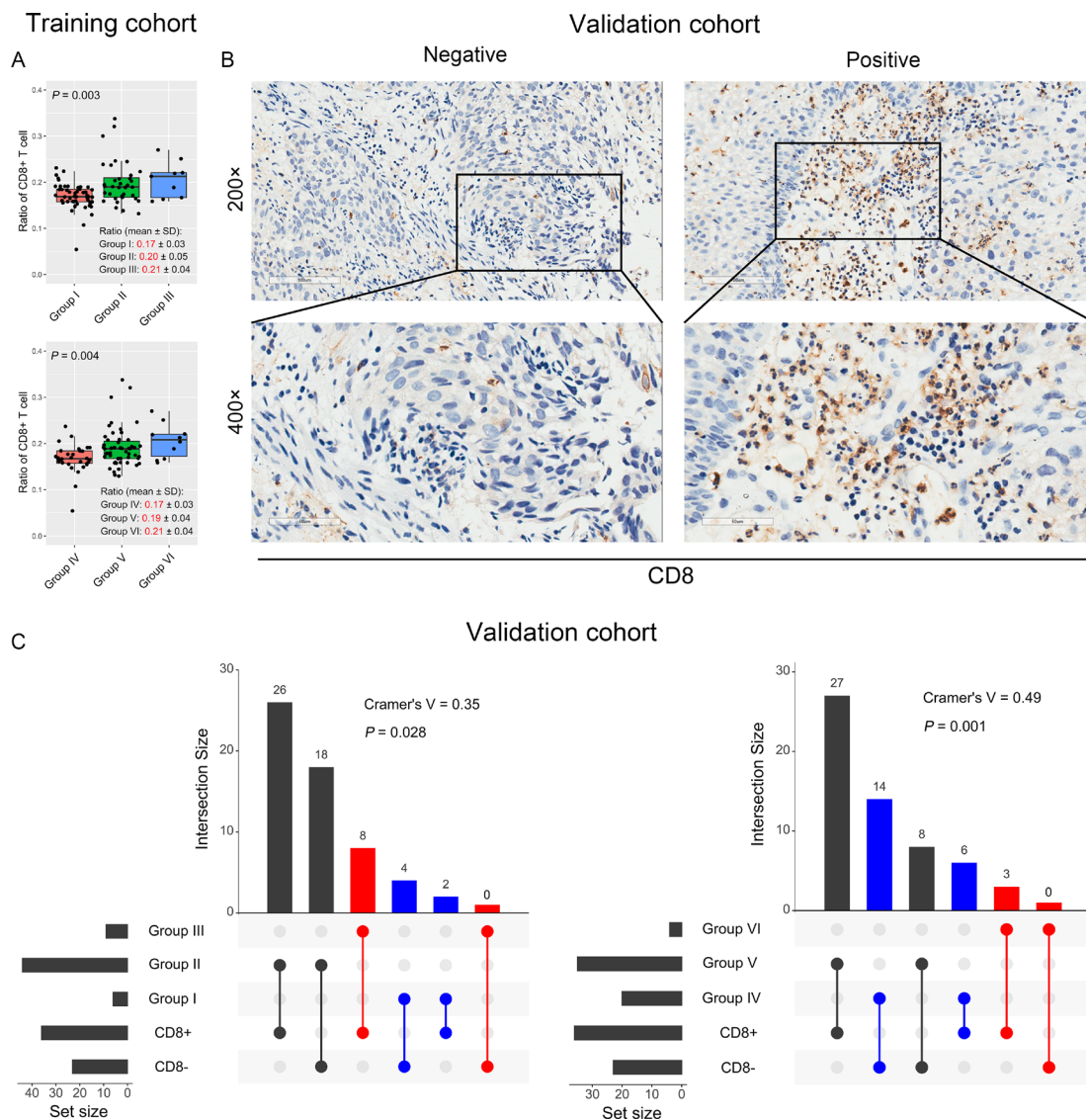
**Figure 6** The relationship between PD-L1/TIM3 or PD-L1/TIGIT and CD8+ T cells. (A) The ratio of CD8+ T cells in different groups of PD-L1/TIM3 (upper panel) or PD-L1/TIGIT (bottom panel) in the training cohorts. The CD4 + and CD8+ T cells ratio in each patient was obtained from the TIMER database (http://cistrome.org/TIMER/). (B) Representative micrographs of CD8 protein expression within the tumor. (C) Evaluation of the relationship between PD-L1/TIM3 (left panel) or PD-L1/TIGIT (right panel) and CD8+ T cells by Cramer's V of $\chi^2$ test in the validation cohort. PD-L1, programmed cell death 1 ligand 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3.

vs 0.15 vs 0.11, p=0.004, online supplemental figure S5B). To validate these findings, we performed IHC to detect CD8 protein expression in ESCC tissues (figure 6B). As expected, PD-L1/TIM3 and PD-L1/TIGIT each had a positive correlation with CD8 protein expression (Cramer's V=0.35, p=0.028; Cramer's V=0.49, p=0.001, respectively) (figure 6C). We further investigated the impact of CD4 and CD8 expression on OS in patients with ESCC and the results suggested that CD4 expression was not significantly correlated with OS (p=0.270, online supplemental figure S6A). Notably, high expression of CD8 was significantly associated with poor OS in the training cohort (HR=2.32, 95% CI: 1.00 to 5.37, p=0.043), though it was not statistically significant in the validation cohort (p=0.397) (online supplemental figure S6B,C).

## Coexpression of PD-1 and TIM3/TIGIT were detected on CD4+ and CD8+ TILs

PD-1 is the receptor of PD-L1 and is a biomarker of T-cell exhaustion in various cancers. Furthermore, high PD-1 expression appeared to be correlated with poor OS for patients with ESCC in the training cohort, though the data were not yet significant enough at this point (p=0.055, online supplemental figure S2A). Therefore, we further explored the coexpression pattern of PD-1 and TIM3/TIGIT on CD4 + and CD8+ TILs in eight ESCC patients with high PD-L1 protein expression by multiplexed immunofluorescence in the validation cohort. Interestingly, the percentages of PD-1 and TIM3/TIGIT coexpression on CD8 + TIL were 75% (6/8) and 37.5% (3/8), respectively (figure 7A). However, the percentages of PD-1 and TIM3/
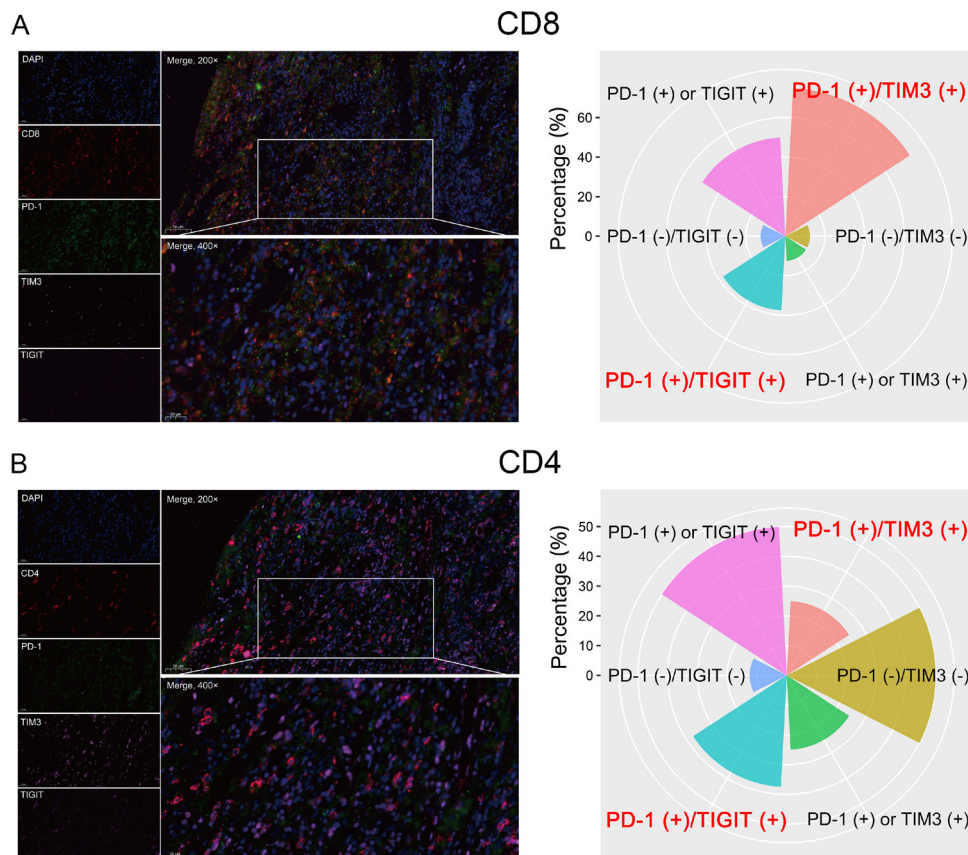
**Figure 7** Detection of PD-1 and TIM3/TIGIT expression on CD8+ (A) and CD4+ (B) cells by multiplexed immunofluorescence. A representative figure shows the expression of CD4, CD8, PD1, TIM3, and TIGIT in a patient with ESCC (left panel). The percentage of PD-1 and TIM3/TIGIT coexpression on CD8+ and CD4+ cells in patients with ESCC (right panel). ESCC, esophageal squamous cell carcinoma; PD-1, programmed cell death 1; TIGIT, T-cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM3, T-cell immunoglobulin and mucin-domain-containing-3.

TIGIT coexpression on CD4 + TIL were only 25% (2/8) and 37.5% (3/8), respectively (figure 7B). Herein, PD-1 and TIM3/TIGIT were primarily coexpressed on CD8+ TIL in patients with ESCC.

## DISCUSSION

Esophageal cancer is an aggressive disease with a poor prognosis. ESCC and esophageal adenocarcinoma are two main histological subtypes. In China, most patients suffer from ESCC. The effects of conventional therapies, including surgery, chemotherapy, and radiotherapy, are unsatisfactory,[19] novel treatments are urgently needed. In recent years, novel prognostic factors of patients with cancer have emerged, especially immune-related gene signatures.[14 17] Immunotherapy with ICBs is promising for ESCC treatment, but how to screen the beneficiaries is still an important issue that needs to be explored.[4]

PD-L1 expression is one of the most useful predictive biomarkers for ICBs and PD-L1 blockade is established as standard of care in patients with ESCC.[4 20] Another checkpoint, TIM3, suppresses antitumor immunity by mediating T-cell exhaustion. It serves as a promising target for cancer immunotherapy.[21] Additionally, the TIGIT pathway can regulate T-cell-mediated tumor recognition

and clinical trials for TIGIT antibodies are ongoing.[22] All are potential immunotherapy targets; however, the integrative expression and predictive values of these checkpoints are little known. In this study, 95 patients with ESCC from the TCGA database and 58 tissue samples were used for OS analysis and validation. Patients with higher expression of PD-L1, TIM3, and TIGIT had a lower OS rate. Our results support the development of inhibitors against these targets in ESCC treatment.

In this study, we found PD-L1 was positively associated with either TIM3 or TIGIT. Coexpression of PD-L1/TIM3 or PD-L1/TIGIT was closely related to the poor OS in patients with ESCCs, especially those with males, younger than 60 years, and TNM III/IV, and either combination was independently prognostic (figure 8). Our nomogram model constructed by PD-L1, TIM3, TIGIT, and TNM stages concisely and precisely predicted ESCC patients' prognosis. These findings suggested the combination of PD-L1/TIM3 or PD-L1/TIGIT as immune biomarkers and also targeted for ICB therapy. In support of our viewpoint, the coexpression of two ICs predicted the prognosis of patients with acute myeloid leukemia.[17]

Due to the limited clinical activity of ICB monotherapy, the combination of ICBs is actively pursued.[4 23] Existing
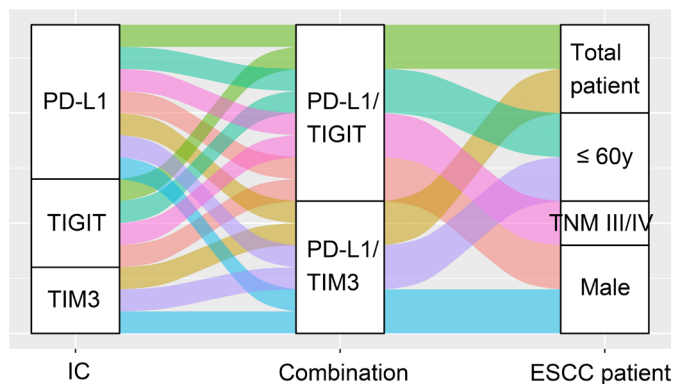
**Figure 8** Schematic summary of PD-L1 and TIM3/
TIGIT combination for OS analysis in patients with ESCC.
ESCC, esophageal squamous cell carcinoma; IC, immune
checkpoint; PD-L1, programmed cell death 1 ligand 1; TIGIT,
T-cell immunoglobulin and immunoreceptor tyrosine-based
inhibitory motif domain; TIM3, T-cell immunoglobulin and
mucin-domain-containing-3; TNM, primary tumor, regional
lymph node, distant metastasis.

evidence suggested that PD-1/PD-L1 and CTLA4 dual
blockade improved the immune response, but at the
price of toxicities.[24] Optimized combinations are urgently
needed. A study showed that the combination of TIM3 and
PD-1/PD-L1 blockades could reverse T-cell exhaustion
in patients with melanoma.[25] Moreover, the synergistic
blockade of PD-L1 and TIGIT can significantly eliminate
tumor cells.[26] In ESCC, a combination of PD-L1/TIM3 or
PD-L1/TIGIT blockade may be helpful.

TILs inside the tumor microenvironment are the key
to tumor eradication. Expression of ICs leads to exhaus-
tion of T cells,[9] while these checkpoints are induced
by immune regulatory factors from TILs.[11 27 28] In vitro
evidence showed CD8+ T cells significantly upregulated
the expression of PD-L1 on tumor cell lines.[12 29] TIM3 is
positively correlated with CD8+ TIL[11] and the combined
blockade of PD-1/PDL1 and Tim-3/galectin-9 can prevent
exhaustion of CD8+ T cells in hematological malignan-
cies.[30] TIGIT+CD8+ T cells infiltration predicts the poor
prognosis and immune escape of patients with cancer.[28]
The synergistic blocking of TIGIT and PD-L1 mainly acts
through CD8+ T cells.[26] In this study, high expression of
CD8 was associated with poor OS in patients with ESCC,
which might be due to the T-cell exhaustion by upreg-
ulating ICs. Interestingly, PD-L1/TIM3 or PD-L1/TIGIT
had a positive correlation with CD8+ T cells. Further-
more, PD-1 and TIM3/TIGIT were primarily coex-
pressed on CD8+ T cells in patients with ESCC. Hence,
it is reasonably inferred that the synergistic blockade of
either PD-L1/TIM3 or PD-L1/TIGIT reverses the inhibi-
tory phenotypes of TILs. Accumulating data are needed
to confirm the negative regulatory effects of coexpression
of PD-L1/TIM3 or PD-L1/TIGIT in patients with ESCC.

This study had limitations. One limitation was no
patients in our study were exposed to ICB treatment
and the impact of ICB treatment could not be evaluated.
Also, the relatively small sample size restricted any further

analysis. Besides, as a retrospective study, bias was almost
inevitable. The conclusion of our study should be consid-
ered with caution.

## CONCLUSION
To our best knowledge, we revealed for the first time
that PD-L1/TIM3 and PD-L1/TIGIT were the optimal
combinations for predicting the prognosis of patients
with ESCC, which might be potential immune targets for
developing combined ICBs therapy.

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