

## Original Article

# Expression of PD-1 on CD8<sup>+</sup> T cells from the peripheral blood of patients with esophageal squamous cell carcinoma and its clinical implications

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**Abstract:** Background: Esophageal cancer is one of the most common gastrointestinal tumors worldwide with poor prognosis and its prognostic evaluation is still an urgent problem. Materials and Methods: Our study was performed to determine the expression of programmed death-1 (PD-1) on CD8<sup>+</sup> T cells from the peripheral blood of patients with esophageal squamous cell carcinoma (ESCC) and to discuss its biological and clinical significance. T-lymphocyte subpopulations in the peripheral blood and PD-1 expression on the surface of CD8<sup>+</sup> T lymphocytes from 60 patients with ESCC (ESCC group) and 25 healthy controls (control group) were counted and analyzed. Results: The results revealed that the percentages of CD3<sup>+</sup> T and CD4<sup>+</sup> T cells, as well as the CD4<sup>+</sup>/CD8<sup>+</sup> T cells ratio, were significantly lower in the ESCC group than that in the control group, whereas the difference in CD8<sup>+</sup> T cells between the ESCC group and the control group did not reach statistical significance. The positive rates of PD-1 expressed in CD8<sup>+</sup> T cells from the ESCC group's peripheral blood were remarkably higher than those from the control group, which was related to tumor cell differentiation and lymph node status but not to sex, age or tumor location. Patients with high PD-1 expression have significantly lower rates of both progression free survival (PFS) and overall survival (OS) compared with those of the low PD-1 expression group; high PD-1 expression was also an independent prognostic factor in patients with ESCC. Conclusion: These findings suggest that T-lymphocyte subpopulations in the peripheral blood of patients with ESCC conferred immunosuppression to varying degrees. PD-1 expression level on the surface of CD8<sup>+</sup> T cells provides a prognostic value of ESCC patients.

**Keywords:** Programmed death 1, esophageal squamous cell carcinoma, peripheral blood, CD8<sup>+</sup> T cell

## Introduction

Esophageal cancer is one of the major causes of gastrointestinal tumors and contributes a great tumor burden worldwide. China contributed almost 1/2 of the new esophageal cancer patients worldwide in 2012, and ESCC was the dominant histological type [1, 2]. Despite advances in diagnosis and treatment, the prognosis for ESCC is still not optimistic because the overall five-year survival is <30% [3]. Therefore, to identify the risk factors and prognostic markers to help find new therapies for esophageal cancer is indispensable.

Current studies have documented that tumor cells can escape the immune response of the

host defense system from death [4]. In recent years, the role of costimulatory molecules in the tumor immune response has become a great concern, and the newly discovered B7 family of co-stimulated molecular PD-1 has been confirmed to be an important molecule that mediates tumor immune escape [5, 6].

PD-1 (CD279) is an inhibitory receptor expressed on various immune cells including T cells, B cells, and myeloid cells, and it's also belong to one of the members of the CD28 family [7]. Recent studies have indicated that PD-1 is also a crucial factor responsible for peripheral tolerance, acting as a negative regulator of the immune system [8]. PD-1 inhibits T-cell activity by transmitting a negative signal, which eventu-

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**Table 1.** Characteristics of 60 ESCC patients and the association between PD-1 expression on CD8<sup>+</sup> T and clinical parameters in ESCC

Variable	CD8 <sup>+</sup> PD-1 <sup>+</sup> T % expression		$\chi^2$	P values
	Low	High		
Sex			3.126	0.077
Male	18	23		
Female	13	6		
Age (years)			1.632	0.201
<70	12	16		
≥70	19	13		
Stage			0.558	0.454
II	18	15		
III	13	14		
Tumor location			1.043	0.594
Upper	5	7		
Middle	22	20		
Lower	4	2		
Differentiation degree			18.038	<0.01*
G1	10	2		
G2	20	13		
G3	1	14		
N stage			22.280	<0.01*
N0	28	9		
N1+N2	3	20		
Treatments			0.918	0.337
Concurrent chemoradiotherapy	21	22		
Radiation therapy	10	7		
Total	31	29		

\*P<0.05 was considered significant.

ally attenuates T lymphocyte activation and proliferation, suppresses the secretion of cytokine, induces T lymphocytes apoptosis and maintains peripheral tolerance [9, 10]. The recent success of using reagents targeting negative regulators of the immune checkpoint protein PD-1 offers great promise for effective cancer therapy [11, 12]. Nivolumab and pembrolizumab, which are agents that block the PD-1 pathway, have been approved by the FDA for treating several solid tumor types [13].

However, the study of PD-1 in the peripheral blood of ESCC patients is limited, and the relationship between clinicopathological features and ESCC remains unclear. It is important to identify prognostic and predictive biomarkers associated with ESCC to guide clinical decisions. Here, we analyzed 60 blood samples of

patients with newly diagnosed ESCC and 25 cancer-free controls. We utilized flow cytometry to evaluate the peripheral expression of PD-1 in CD8<sup>+</sup> T cells and further dissect their prognostic and predictive value in ESCC.

### Materials and methods

#### Patients and controls

A total of 60 patients (41 men and 19 women) with newly diagnosed stage II or III ESCC and 25 cancer-free controls were enrolled in our study; their median age was 70 years (range, 52-84). Patients who received any prior neoadjuvant treatment or those with immune system diseases were excluded. All patients were recruited between January and June 2015 at the First Affiliated Hospital of Bengbu Medical College. The primary tumors of ESCC were diagnosed by the pathology department of the First Affiliated Hospital of Bengbu Medical College. Evaluation of tumor differentiation was based on histologic criteria following the World Health Organization's guidelines [14]: 12 (20%) were well differentiated, 33 (55%) were moderately differentiated, and 15 (25%) were poorly differentiated. Moreover, 22 (37%) cases

had lymphatic metastasis, and 38 (63%) cases did not. The patients were treated with definitive radiotherapy or chemoradiotherapy because the disease was not amenable to resection, patients had multiple medical comorbidities that would preclude surgery or the patient declined surgery. The control group was selected from the same period from the hospital physical examination center and was gender and age-matched; health examinations were performed for 25 subjects, including 16 males and 9 females. Their median age was 68 years (range, 55-78). The characteristics of 60 ESCC patients and controls are shown in **Table 1**. A total of 54 of 60 patients' complete follow-up records were available during 24 months. All of this study's protocols were approved by the Board of Ethics of the First Affiliated Hospital of Bengbu Medical College, and all the patients

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**Table 2.** The expression of peripheral blood T lymphocyte subsets from ESCC group and control group

	ESCC (%)	Controls (%)	t value	P value
CD3 <sup>+</sup>	59.2±1.7	67.5±2.5	2.72	<0.01*
CD4 <sup>+</sup>	32.2±1.2	41.1±1.8	4.01	<0.01*
CD8 <sup>+</sup>	25.1±1.4	22.7±1.9	0.91	0.36
CD4 <sup>+</sup> /CD8 <sup>+</sup>	1.2±0.4	1.8±0.52	2.51	0.02*

\*P<0.05 was considered significant.

enrolled signed an informed consent form before entering the study.

### Sample collection and processing

A total of 4 ml of peripheral blood was collected in an EDTA-K2 vacutainer from each patient before any treatment. After collection, the specimens were stored at 4°C and were evaluated within 6 h. Then serum was extracted and reserved in EP tube at the temperature of -80°C after limosis vein blood was centrifuged at the speed of 2000 r/min for 10 minutes (centrifugal radius was 8 cm). Operating steps followed the kit instructions. The methods of collection and preparation of specimen from ESCC patients (before therapy) and normal controls were the same.

### Flow cytometry

The number of T-lymphocyte subpopulations in the peripheral blood and PD-1 expressed on CD8<sup>+</sup> T cells were assessed by flow cytometry (BD FACSCalibur, Becton Dickinson, San Jose, CA, USA) in all studied cases. Firstly, 100 µl of EDTA-anticoagulated whole blood was stained with 5 µl of CD3/CD8/CD45/CD4 multitest antibodies (BD Biosciences) and incubated for 30 minutes at room temperature, in dark. Thereafter, the cells were lysed with the use of BD FACS Lysing Solution (BD Biosciences), washed twice with cold phosphate buffered saline (Biomed Lublin, Poland). Stained cells in each sample was performed using the CellQuest software (Becton Dickinson) [15]. Secondly, peripheral blood mononuclear cells were suspended in PBS containing 20% human AB serum and incubated for 30 min on ice with appropriate dilution of antibodies. The antibodies used in this part were as follows: anti-CD3-APC, anti-CD8-FITC, and anti-CD279 (PD-1)-PE (all from BioLegend). For the negative controls, APC mouse IgG1 (j) (clone MOPC-21; BD Bio-

sciences) and PE mouse IgG2b (j) (clone MPC-11; BioLegend) were used. Specimen acquisition was performed using the CellQuest software (Becton Dickinson).

### Statistical analysis

GraphPad Prism 5 (San Diego, USA) was used for graphic creation and statistical analysis. A two-tailed Student's t test was used to analyze the differences among two groups. The log-rank test was used to analyze the statistic differences between the PD-1<sup>+</sup> % low vs. PD-1<sup>+</sup> % high patients. Pearson  $\chi^2$  test or Fisher's exact test were assessed to correlations of PD-1 expression and other clinicopathological characteristics. Kaplan-Meier survival curves were used to evaluate the PFS and OS. In addition, a Cox proportional hazards regression model was developed to find potential prognostic factors for survival. A value of  $p<0.05$  was considered a significant difference.

## Results

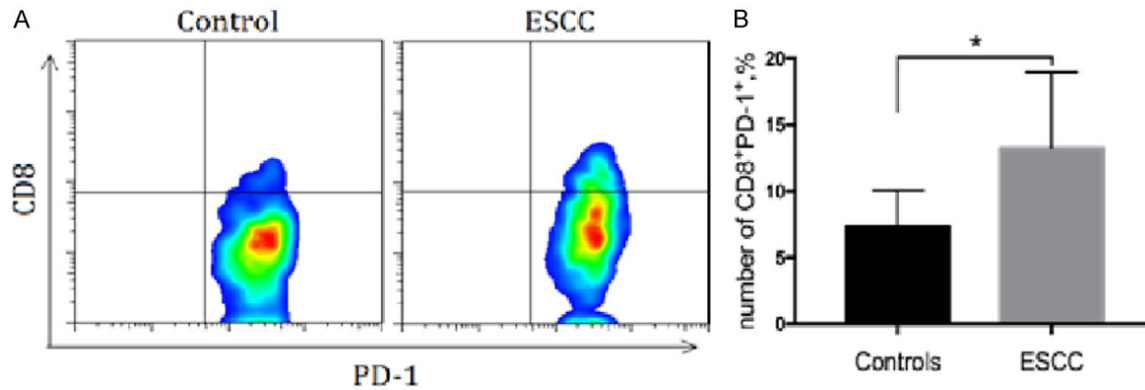
### Comparison of T-lymphocyte subpopulations from the peripheral blood of ESCC and control groups

The results of the comparison between peripheral blood T-lymphocyte subsets from ESCC cases and controls are presented in **Table 2**. The percentages of CD3<sup>+</sup> T and CD4<sup>+</sup> T lymphocyte subsets and the CD4<sup>+</sup>/CD8<sup>+</sup> T cells ratio in the ESCC group were significantly lower than those in the controls ( $P<0.05$ ). CD8<sup>+</sup> T lymphocyte subsets in the ESCC group was higher percentage than that in the controls, but the difference did not reach statistical significance ( $P = 0.36$ ).

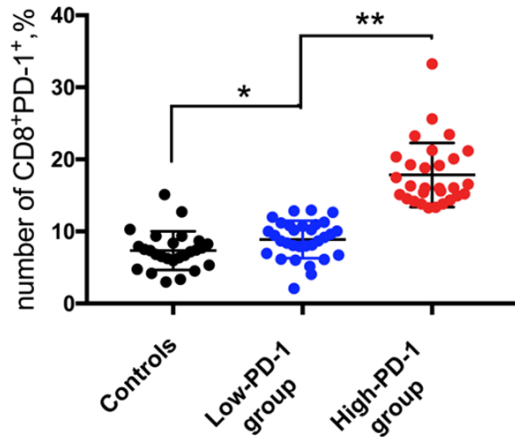
### PD-1 is elevated on CD8<sup>+</sup> T cells from the peripheral blood of patients with ESCC

We assessed PD-1 expression on CD8<sup>+</sup> T cells at initial diagnosis using peripheral blood from 60 ESCC patients and 25 healthy controls. All 60 patients met the clinical videography and histological criteria for ESCC stage II or III (**Table 1**). As shown in **Figure 1**, a higher proportion of PD-1<sup>+</sup>CD8<sup>+</sup> T cells was detected in the ESCC group than that in the healthy controls (mean  $\pm$  standard error of the mean,  $13.2\pm0.7\%$  vs.  $7.3\pm0.5\%$ ,  $P<0.01$ ). Based on the level of PD-1 expression on CD8<sup>+</sup> T cells, we defined high-PD-1 (PD-1>13.34%) vs. low-PD-1 (PD-1<13.34%) subgroups in the ESCC patients.

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**Figure 1.** 1 million of peripheral blood mononuclear cells from ESCC patients or normal donors were used for quantification of PD-1<sup>+</sup> % in CD3<sup>+</sup>CD8<sup>+</sup> T cells by flow cytometry. PD-1 expression in CD8<sup>+</sup> T cells is higher in ESCC patients than ones in healthy controls. Statistical significance was determined by a Student t test (\*,  $p < 0.05$ ).



**Figure 2.** PD-1 expression levels on the surface of CD8<sup>+</sup> T cells of ESCC patients with different expression levels. \* $P = 0.025$ , compared to the control group. \*\* $P < 0.01$ , compared to the Low-PD-1 group.

The mean value (13.34%) of PD-1 expression on CD8<sup>+</sup> T cells for 60 ESCC patients was the cutoff [16]. 29 patients were classified in the high-PD-1 group and 31 in the low-PD-1 group. The rates of PD-1 on the surface of CD8<sup>+</sup> T cells in the high-PD-1 group were significantly higher than those in the low-PD-1 group ( $17.9 \pm 0.8\%$  vs.  $8.9 \pm 0.5\%$ ,  $P < 0.01$ , **Figure 2**). However, the differences in PD-1 expression were not statistically significant between the low-PD-1 group and the control group ( $8.9 \pm 0.5\%$  vs.  $7.3 \pm 0.5\%$ ,  $P = 0.25$ , **Figure 2**).

*High expression of PD-1 correlates with tumor cell differentiation degree and lymph node metastasis*

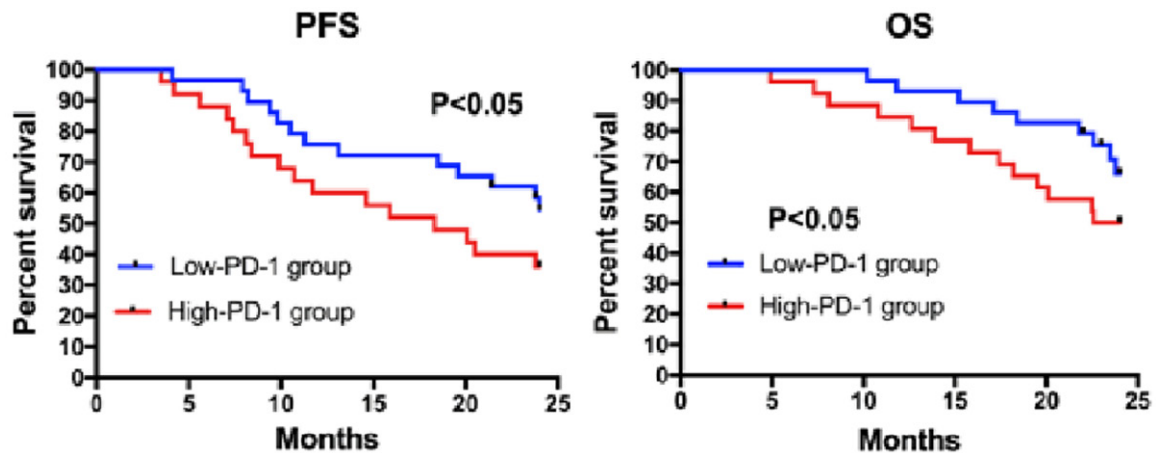
The correlation of PD-1 expression with the clinical characteristics of ESCC patients is

shown in **Table 1**. The frequency of high PD-1 expression in poorly differentiated patients was 93.3% (14/15), significantly higher than the 33.3% (15/45) for moderate to high differentiation ( $\chi^2 = 18.038$ ;  $P < 0.01$ ); the frequency of high PD-1 expression in cases with lymphatic metastasis was 86.9% (20/23), higher than the 21.6% (8/37) of cases without lymphatic metastasis ( $\chi^2 = 22.280$ ,  $P < 0.01$ ). However, no correlation was found between the PD-1 expression on peripheral blood CD8<sup>+</sup> T cells and the sex, age or tumor location of ESCC patients.

*High PD-1 expression on CD8<sup>+</sup> T cells at initial diagnosis is associated with poor prognosis and is an independent prognostic factor in predicting a poor clinical outcome in ESCC patients*

We analyzed clinical characteristics between the high and low PD-1 groups in terms of age, gender, stage and non-surgical treatments (concurrent chemoradiotherapy or radiation therapy) and found no significant difference ( $P = 0.337$ , **Table 1**). We next investigated the effects of PD-1 expression on clinical outcomes. Kaplan-Meier survival analysis of the 54 ESCC patients with complete clinical follow-up showed that there is a significantly lower rate of both PFS and OS in the high-PD-1 group compared with the low-PD-1 group. The PFS rates at 1 and 2 years were 58.3 and 39.7%, respectively, for high-PD-1 expressing patients and 76.9% and 60.2%, respectively, for low-PD-1 expressing patients. Similarly, the OS rates at 1 and 2 years were 83.1% and 50.4%, respectively, for high-PD-1 expressing patients and 91.6% and 69.2%, respectively, for low-

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**Figure 3.** Kaplan-Meier curves of 56 patients with ESCC segregated into 2 groups, according to the median value of PD-1<sup>+</sup>CD8<sup>+</sup> % cells (with 13.34% as a threshold). PD-1<sup>+</sup>CD8<sup>+</sup> % cells >13.34% was defined as high-PD-1 group while <13.34% as low-PD-1 group. Kaplan-Meier survival analysis showed there is a significantly lower rate of both PFS and OS in the high-PD-1 group compared with the low-PD-1 group. Statistical significance was determined by a Log-rank (Mantel-Cox) Test.

**Table 3.** Multivariate Cox regression analysis of clinicopathological factors for risk prediction in 54 patients with ESCC

Factor	Risk	95% CI	P value
Age	0.846	0.581-1.231	0.428
Sex	0.765	0.523-1.117	0.173
Tumor location	0.979	0.692-1.383	0.936
Differentiation	0.553	0.342-0.922	0.027*
Lymph node metastasis	2.337	1.667-3.279	<0.01*
Treatments	2.346	1.682-3.275	<0.01*
PD-1 expression	1.786	1.293-2.468	0.023*

\*P<0.05 was considered significant.

PD-1 expressing patients (**Figure 3**). These results demonstrated that high PD-1 expression on peripheral CD8<sup>+</sup> T cells is correlated with worse clinical outcomes in ESCC, independent of age, gender, stage and non-surgical treatments.

The Cox proportional hazards regression model demonstrated that high expression of PD-1 on CD8<sup>+</sup> T cells was also an independent prognostic factor in predicting poor clinical outcome in ESCC patients (risk = 1.786; 95% CI = 1.293-2.468, P = 0.023), in addition to tumor differentiation grade (risk = 0.553; 95% CI = 0.342-0.922, P = 0.027), lymphatic metastasis (risk = 2.337; 95% CI = 1.667-3.279, P<0.001), and treatments (risk = 2.346; 95% CI = 1.682-3.275, P<0.001) (**Table 3**). However, it was not an independent prognostic factor for age, sex,

or tumor location (P = 0.428, 0.173, and 0.936, respectively).

### Discussion

In our research, we evaluated the phenotype of peripheral blood T cells collected from ESCC patients and determined the correlation between PD-1 expression on the CD8<sup>+</sup> T cells with clinical implications and clinical outcomes. First, we investigated the counts of T-lymphocyte subpopulations and the expression of PD-1 on CD8<sup>+</sup> T cells from peripheral blood of patients with ESCC; we identified that T-lymphocyte subpopulations in the peripheral blood conferred immunosuppression to varying degrees and that PD-1 expression on CD8<sup>+</sup> T cells is significantly elevated in ESCC patients at the initial diagnosis. Additionally, our data revealed that the high expression of PD-1 on CD8<sup>+</sup> T cells was further correlated with the tumor cell differentiation degree and lymph node metastasis. In addition, high expression of PD-1 on CD8<sup>+</sup> T cells is strongly associated with a poor clinical outcome manifested by significantly shorter OS and PFS in patients with ESCC; high PD-1 expression was also an independent prognostic factor, and this is prognostic.

Recent success at targeting immune inhibitory pathways has caused great excitement in cancer therapy. PD-1 is a critical receptor expressed on T cells that can mediate immunosuppres-



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sion and the escape from immune system destruction by interacting with its ligands PD-L1 and PD-L2 on tumor cells [17]. Inhibiting the PD-1 pathway can enhance the anti-tumor activity of cytotoxic T cells. Two antibodies that block PD-1, nivolumab and pembrolizumab, have been approved by the FDA for treating advanced solid tumors. PD-1 expression has been found in various human cancers, and its expression level has been significantly associated with patients' prognoses and a series of clinicopathological parameters of the patients, such as lung cancer [18], bladder cancer [19], and gastric cancer [20]. However, little is known about the relationship between the expression of PD-1 in the peripheral blood and ESCC or the significance of PD-1 expression in predicting the prognosis of ESCC patients. It is important to determine the prognostic and predictive biomarkers that identify patients at a high risk of disease progression.

Tumor immunity mainly depends on cellular immunity, in which T lymphocytes are at the center; they play an important role in immune surveillance, killing target cells and participating in immunoregulation. There are different roles of T lymphocyte subtypes in anti-tumor immunity; therefore, detecting peripheral blood T lymphocyte subsets is an important indicator of the body's anti-tumor status. The percentages of CD3<sup>+</sup> T and CD4<sup>+</sup> T cells, as well as the CD4<sup>+</sup>/CD8<sup>+</sup> T cells ratio, were significantly lower in the ESCC group than those in the control group. However, in the ESCC group, the CD8<sup>+</sup> T lymphocyte subsets are greater than those in the controls, but these results did not reach statistical significance. Consequently, these results indicate that patients with ESCC have cell immune function imbalance in their peripheral blood.

PD-1 is usually upregulated in many different tumor types [21], it is a recognized marker of T-cell dysfunction because it inhibits antitumor T cell responses, and blockade of the PD-1 pathway has been used for cancer immunotherapy. In our study, we find that the expression of PD-1 is indeed increased on CD8<sup>+</sup> T cells from the peripheral blood of patients with ESCC. This finding was also seen in several other studies [22-24]. Moreover, the expression of PD-1 is strongly associated with tumor cell differentiation degree and lymphatic

metastasis in tumors. These data suggest that blockade of this pathway is a potential treatment for ESCC. In addition, Kaplan-Meier survival analysis demonstrates that high expression of PD-1 on CD8<sup>+</sup> T cells is associated with a poor clinical outcome, which has significant clinical implications. This may explain why high PD-1 expression in T cells results in an immunosuppressed state and a less effective antitumor response, leading to tumor progression and poor overall clinical outcomes. Moreover, Multivariate Cox regression analysis demonstrates that the high expression of PD-1 is an independent prognostic factor in predicting clinical outcomes in ESCC patients. A study of extrahepatic bile duct cancer showed similar results as to ours [25], in which the ratio of PD-1<sup>+</sup> CD8<sup>+</sup> T cells was found to be an independent prognostic factor. The above data collectively show that PD-1 serves as a biomarker in tumors, as well as a drug target, and its expression levels provide valuable prognostic information for clinical therapy in ESCC patients.

In our study, we used peripheral blood samples from patients with ESCC to demonstrate a negative prognostic value of PD-1 in this population, i.e., high expression of PD-1 is associated with poor PFS and OS; PD-1 was also an independent prognostic factor in ESCC patients. Importantly, we avoided the need for invasive tissue biopsy, which may lead to treatment delays and a potential risk of complications. Flow cytometry is a defined and good quantitative method to determine the level of PD-1 expression on peripheral blood cells. Our study provided a highly feasible and effective clinical method to determine the prognosis of patients with ESCC.

However, it should be noted that this study was retrospective nature with a relatively small sample size limits the generalization of our findings. Further observations with large-scale, multi-center, prospective, randomized clinical trials are needed to verify our discoveries.

In conclusion, our study is the first report of high expression of PD-1 on CD8<sup>+</sup> T cells from the peripheral blood of ESCC patients which demonstrate the prognostic value of PD-1 expression on CD8<sup>+</sup> T cells, while high PD-1 expression is associated with a poor clinical

outcome. This might suggest a potential predictive biomarker for ESCC and provide a possible target for the treatment of this disease.

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## Disclosure of conflict of interest

None.

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