

Research Paper

PD-L1 Expression On tumor Cells Was Associated With Unfavorable Prognosis In Esophageal Squamous Cell Carcinoma

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Received: 2017.12.21; Accepted: 2018.03.26; Published: 2018.06.05

Abstract

Background: Evidence about the association between programmed cell death ligand 1 (PD-L1) expression and prognosis of esophageal squamous cell carcinoma (ESCC) were limited and controversial. Thus, the present study aims to investigate the prognostic value of tumor immune microenvironment (TIM) based on PD-L1 expression and CD8+ T cell infiltration in ESCC tissues.

Methods: From September 2008 to March 2010, a total of 146 ESCC patients received radical esophagectomy were retrospectively analyzed in our present study. PD-L1 expression and CD8+ T cell infiltration were evaluated through immunohistochemistry. The clinicopathological characteristics and survival were analyzed.

Results: There were 111 male and 35 female. The median age was 59.1 years (37-78 years). The positive rate of PD-L1 expression was 61.7%. The rate of high CD8+ T cell infiltration was 33%. No significant differences were found between clinicopathological features and PD-L1 expression or CD8+ T cell infiltration. PD-L1 expression was significantly associated with poor overall survival ($P=0.010$). However, CD8+ T cell infiltration was not a prognostic risk factor. Type of TIM was significantly associated with the prognosis of ESCC patients ($P=0.021$).

Conclusions: PD-L1 expression was an independent risk factor for the prognosis of ESCC patients. Immunotherapy may achieve promising outcomes in ESCC patients with type I TIM.

Key words: esophageal squamous cell carcinoma, programmed cell death ligand 1, tumor immune microenvironment, prognosis

Introduction

ESCC is the sixth most common cancer and is ranked fourth in the cancer related mortality in China. Commonly, the incidence of ESCC is highest in central China, and the clinicopathological features are different from the West [1,2]. Although multidisciplinary therapies have been applied to the treatment of ESCC, the prognosis of patients is still not promising [2].

PD-L1 belongs to the B7 super-family, which is expressed on activated T cells, B cells, dendritic cells, macrophages, and also on tumor cells [3,4]. PD-1 is expressed on the surface of immune cells. The binding of PD-L1/PD-1 could enable tumor cells to avoid antitumor immunity [5]. Thus, the immunotherapy, targeting the PD-L1/PD-1 immune checkpoint, has been an emerging field. Several clinical trials have

shown promising antitumor activity of PD-L1/PD-1 blockade in several malignancies [6-11]. However, not all patients obtained favorable response and durable efficacy [12,13]. This may attribute to the different type of TIM in tumor tissues. Based on the expression of PD-L1 on tumor cells and CD8+ T cell infiltration in tumor tissues, TIM was classified into four types: type I (PD-L1+/CD8 High, adaptive immune resistance), type II (PD-L1-/CD8 Low, immune ignorance type), type III (PD-L1+/CD8 Low, intrinsic induction of PD-L1 in the absence of TILs), and type IV (PD-L1-/CD8 High, components other than PD-L1 suppressing the action of TILs) [14]. It was reported that type of TIM was a potentially powerful biomarker for the prognosis of solid cancers [15], and type I TIM have been demonstrated to be a potential subgroup for anti-PD-1 or anti-PD-L1 immunotherapy [7,16,17].

Recently, a series of studies have investigated the prognostic value of expression of PD-L1 on tumor cells in ESCC patients. However, the findings were controversy. Most studies reported that PD-L1 expression was associated with poor prognosis of ESCC patients [18-20]. However, a few investigations reported the opposite results [21,22]. Up to date, the prognostic value of TIM in ESCC patients has not been investigated yet. Thus, the present study co-assessed PD-L1 expression and CD8+ T cell infiltration in ESCC, and investigated the prognostic value of PD-L1 expression and type of TIM in ESCC.

Materials and methods

Patients

This study was performed in the Xijing Hospital of Digestive Diseases affiliated to the Fourth Military Medical University. From September 2008 to March 2010, a total of 146 ESCC patients were enrolled in our present study. The inclusion criteria were listed as follows: 1. treated with radical esophagectomy, 2. with follow up data. The exclusion criteria were: 1. with neoadjuvant chemotherapy, 2. with distant metastasis, 3. with other malignancies. The clinicopathological data including age, gender, tumor location, tumor size, differentiation status, tumor depth, lymph node metastasis and TNM stage were recorded. The tumors were staged according to the seventh edition of the American Joint Committee on Cancer TNM classification. The patients were followed up until November 2016 by enhanced CT every 3 months. This study was approved by the Ethics Committee of Xijing Hospital, and written informed consent was obtained from all patients before surgery.

Immunohistochemistry

The tissues were fixed with 4% formaldehyde, embedded in paraffin and sectioned serially at 4 μ m thickness. Briefly, the sections were deparaffinized in xylene, dehydrated with graded ethanol, pretreated in citrate buffer with 2 minutes in a pressure cooker for antigen retrieval, and then blocked with 3% H₂O₂ for 10 mins, goat serum for 10 mins. After blocking, the sections were incubated with rabbit anti-PD-L1 monoclonal antibody (1:200, ab13684S, Cell Signaling Technology, USA) or anti-CD8+ monoclonal antibody (1:50, clone: C8/144B, ZSDB, China) at 4°C overnight, and then incubated with HRP-Polymer anti-rabbit IHC Kit (Fuzhou Maixin Biotechnology, China) at room temperature for 30 minutes according to the manufacturer's instructions.

Evaluation of immunostaining

All tissue slides were evaluated by 3 independent pathologists. The immunoreactivity scoring system (IRS) was calculated based on the intensity category and percentage category. The intensity category of immunostaining was graded as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The percentage category was graded as follows: 0 (negative), 1 (1%-30%), 2 (31% to 60%) and 3 (61%-100%). The IRS was calculated by multiplication of both categories [23]. Then, the expression of PD-L1 was classified as negative ($0 \leq IRS \leq 2$) and positive ($3 \leq IRS \leq 9$) based on IRS. In order to ensure the quality control during IHC evaluation, positive and negative control was used during IHC to exclude nonspecific staining [24-27]. Tissue slides were evaluated by 3 independent pathologists who were blinded to clinical outcomes. When the results were inconsistent, the results were discussed by the 3 independent pathologists to make a final evaluation. The numbers of CD8+ T cell were counted in 6-10 high power magnification (40x) field with the most abundant distribution of CD8+ cells within tumor. The average numbers of CD8+ T cell per high power magnification field (HPF) were recorded. The CD8 high and CD8 low groups were defined using the 66th percentile of the average as the cut-off value. Based on density of CD8+ T cell and expression of PD-L1, type of TIM was classified into four types: type I (PD-L1+/CD8 high), type II (PD-L1-/CD8 low), type III (PD-L1+/CD8 low) and type IV (PD-L1-/CD8 high).

Statistical analysis

Data were processed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA). Discrete variables were analyzed using Chi-square test or Fisher's exact test. Significant risk factors for the prognosis of ESCC patients identified by univariate

analysis were further assessed by multivariate analysis using the Cox's proportional hazards regression model. Overall survival was analyzed by Kaplan-Meier method. The P value was considered to be statistically significant at 5% level.

Table 1. Comparison of clinicopathological features of ESCC patients according to PD-L1 expression and CD8+ cell infiltration.

Characteristics	PD-L1-	PD-L1+	P	CD8 low	CD8 high	P
Gender						
Male	38(34.2%)	73(65.8%)	0.068	72(64.9%)	39(35.1%)	0.678
Female	18(51.4%)	17 (48.6%)		24(68.6%)	11(31.4%)	
Age						
≤60	31(39.7%)	47(60.3%)		52(66.7%)	26(33.3%)	0.803
>60	25(36.8%)	43(63.2%)		44(64.7%)	24(35.3%)	
Tumor location						
Upper third	12(42.9%)	16(57.1%)	0.844	21(75.0%)	7(25.0%)	0.271
Middle third	27(38.0%)	44(62.0%)		48(67.6%)	23(32.4%)	
Lower third	17(36.2%)	30(63.8%)		27(57.4%)	20(42.6%)	
Tumor size (cm)						
≤3	24(46.2%)	28(53.8%)	0.155	34(65.4%)	18(34.6%)	0.718
3<, ≤5	20(40.8%)	29(59.2%)		34(69.4%)	15(30.6%)	
>5	12(27.3%)	32(72.7%)		27(61.4%)	17(38.6%)	
Differentiation status						
Well	32(43.2%)	42(56.8%)	0.312	53(71.6%)	21(28.4%)	0.029
Moderately	19(36.5%)	33(63.5%)		35(67.3%)	17(32.7%)	
Poorly	5 (25.0%)	15(75.0%)		8(40.0%)	12(60.0%)	
Tumor depth						
T1	8 (50.0%)	8(50.0%)	0.504	8(50.0%)	8(50.0%)	0.059
T2	17(35.4%)	31(64.6%)		36(75.0%)	12(25.0%)	
T3	31(38.8%)	49(61.3%)		52(65.0%)	28(35.0%)	
T4	0 (0%)	2 (100%)		0(0%)	2(100%)	
Lymph node metastasis						
N0	34(42.0%)	47(58.0%)	0.308	53(65.4%)	28(34.6%)	0.085
N1	21(37.5%)	35(62.5%)		38(67.9%)	18(32.1%)	
N2	1 (25.0%)	37 (5.0%)		4(100%)	0(0%)	
N3	0 (0%)	5(100%)		1(20.0%)	5(80.0%)	
TNM stage						
I	14(45.2%)	17(54.8%)	0.570	18(58.1%)	13(41.9%)	0.219
II	26(38.8%)	41(61.2%)		49(73.1%)	18(26.9%)	
III	16(33.3%)	32(66.7%)		29(60.4%)	19(39.6%)	

Results

Clinicopathologic features, PD-L1 expression and CD8+ T cell infiltration

There were 111 male and 35 female. The median age was 59.1 years (37-78 years). The positive rate of PD-L1 expression was 61.7%. The median follow-up time was 35.9 months (0.83-77.3 months). Eighty-eight patients died (60.2%). Median overall survival was 31.2 months. The median CD8+ T cell density was 11.8/HPF (0-58/HPF), and the cut-off value of density was 13/HPF. The association between clinicopathological features and PD-L1 expression and CD8+ T cell infiltration were summarized in Table 1. No significant differences were found between clinicopathological features and PD-L1 expression (all P>0.05). For CD8+ T cell infiltration, only differentiation status was significantly associated with CD8+ T cell infiltration (P=0.029).

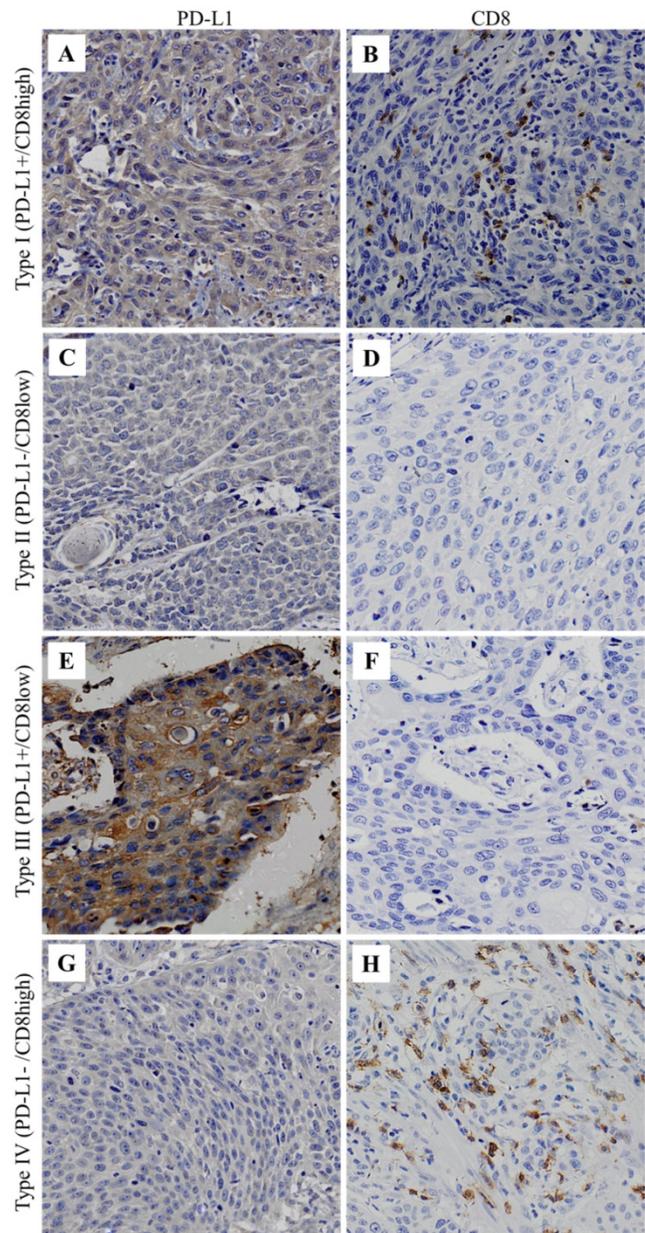


Figure 1. Type of TIM in ESCC tissues (×100). A and B, the same patient with type I TIM. C and D, the same patient with type II TIM. E and F, the same patient with type III TIM. G and H, the same patient with type IV TIM.

TIM classification based on PD-L1 expression and CD8+ T cell infiltration

The TIM of ESCC were classified into four types according to PD-L1 expression and CD8+ T cell infiltration (Figure 1). The distribution of the four types was 22.0% for type I, 26.0% for type II, 39.7% for type III and 12.3% for type IV. The associations between clinicopathological features and type of TIM were shown in Table 2. The results showed that only differentiation status was significantly associated with TIM (P=0.001).

Table 2. Association between clinicopathological features and tumor immune microenvironment.

Characteristics	PD-L1+, CD8 high	PD-L1-, CD8 low	PD-L1+, CD8 low	PD-L1-, CD8 high	P
Gender					
Male	26(23.4%)	25(22.5%)	47(42.3%)	13(11.7%)	0.307
Female	6(17.1%)	13(37.1%)	11(31.4%)	5(14.3%)	
Age					
≤60	16(20.5%)	21(26.9%)	31(39.7%)	10(12.8%)	0.972
>60	16(23.5%)	17(25.0%)	27(39.7%)	8(11.8%)	
Tumor location					
Upper third	4(14.3%)	9(32.1%)	12(42.9%)	3(10.7%)	0.428
Middle third	17(23.9%)	21(29.6%)	27(38.0%)	6(8.5%)	
Lower third	11(23.4%)	8(17.0%)	19(40.4%)	9(19.1%)	
Tumor size (cm)					
≤3	11(21.2%)	17(32.7%)	17(32.7%)	7(13.5%)	0.510
3<, ≤5	8(16.3%)	13(26.5%)	21(42.9%)	7(14.3%)	
>5	13(29.5%)	8(18.2%)	19(43.2%)	4(9.1%)	
Differentiation status					
Well	11(14.9%)	22(29.7%)	31(41.9%)	10(13.5%)	0.001
Moderately	9(17.3%)	11(21.2%)	24(46.2%)	8(15.4%)	
Poorly	12(60.0%)	5(25.0%)	3(15.0%)	0(0%)	
Tumor depth					
T1	4(25.0%)	4(25.0%)	4(25.0%)	4(25.0%)	0.259
T2	7(14.6%)	12(25.0%)	24(50.0%)	5(10.4%)	
T3	19(23.8%)	22(27.5%)	30(37.5%)	9(11.3%)	
T4	2(100%)	0(0%)	0(0%)	0(0%)	
Lymph node metastasis					
N0	16(19.8%)	22(27.2%)	31(38.3%)	12(14.8)	0.362
N1	12(21.4%)	15(26.8%)	23(41.1%)	6(10.7%)	
N2	0(0%)	1(25.0%)	3(75.0%)	0(0%)	
N3	4(80.0%)	0(0%)	1(20.0%)	0(0%)	
TNM stage					
I	5(16.1%)	6(19.4%)	12(38.7%)	8(25.8%)	0.153
II	13(19.4%)	21(31.3%)	28(41.8%)	5(7.5%)	
III	14(29.2%)	11(22.9%)	18(37.5%)	5(10.4%)	

Table 3. Univariate analysis of risk factors for overall survival of ESCC patients.

Prognostic factors	β	HR (95% CI)	P value
Gender	-0.394	0.674(0.397-1.145)	0.145
Age	0.044	1.045(0.688-1.588)	0.835
Tumor location	-0.065	0.937(0.689-1.258)	0.667
Tumor size	0.073	1.076(0.830-1.394)	0.580
Differentiation status	0.171	1.186(0.880-1.597)	0.262
Tumor depth	0.547	1.727(1.224-2.438)	0.002
Lymph node metastasis	0.439	1.551(1.181-2.036)	0.002
TNM stage	0.518	1.678(1.242-2.267)	0.001
PD-L1 expression	0.589	1.803(1.147-2.834)	0.011
CD8+ cell infiltration	0.122	1.130(0.732-1.746)	0.581

Table 4. Multivariate analysis of risk factors for prognosis of ESCC patients.

Prognostic factors	β	HR (95% CI)	P value
Tumor depth	0.461	1.586(1.108-2.269)	0.012
Lymph node metastasis	0.273	1.314(0.985-1.753)	0.063
PD-L1 expression	0.496	1.643(1.038-2.601)	0.034

Survival analysis

The 1, 3 and 5 years overall survival was 76.0%, 45.9% and 39.7%, respectively. The risk factors for the prognosis of ESCC patients were analyzed using univariate analysis (Table 3). The results showed that tumor depth, lymph node metastasis, TNM stage and

PD-L1 expression were risk factors for the prognosis of ESCC patients (all P<0.05). However, only tumor depth and PD-L1 expression were independent prognostic risk factors (both P<0.05). The overall survival of ESCC patients according to PD-L1 expression was shown in Figure 2. The prognosis of patients with positive PD-L1 expression was significantly lower than that with negative PD-L1 expression. The overall survival of patients stratified by type of TIM were also analyzed and shown in Figure 3. The 5-year overall survival of Type I, II, III and IV was 24.7%, 40.8%, 32.3% and 50.2%, respectively.

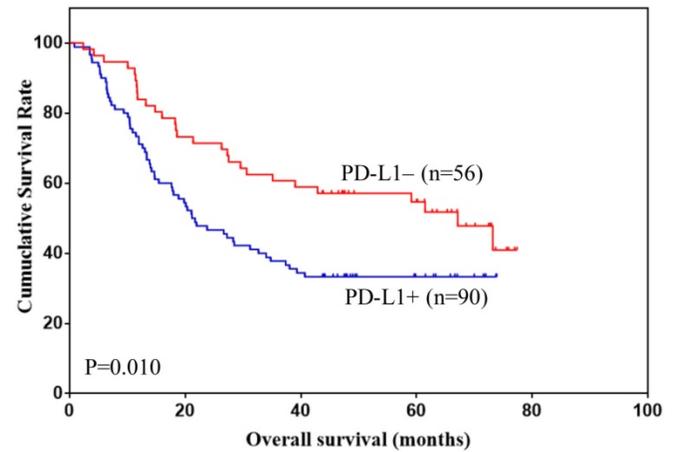


Figure 2. Overall survival according to the PD-L1 expression on tumor cells.

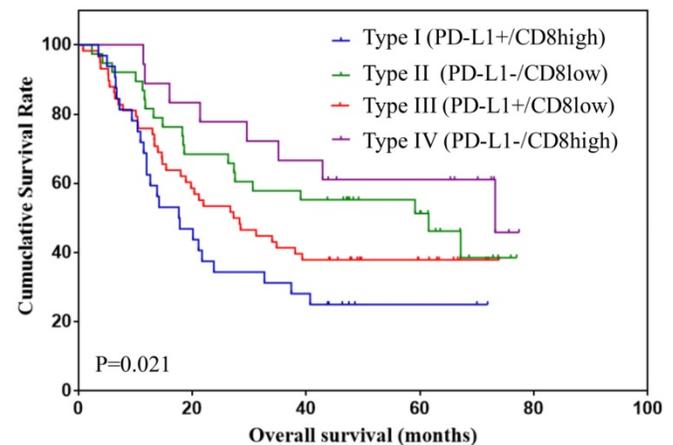


Figure 3. Overall survival according to type of TIM.

Discussion

Although the associations between PD-L1 expression on tumor cells and prognosis of ESCC patients have been investigated in a series of studies, the findings were limited and controversial. Thus, the present study aims to investigate the prognostic value of PD-L1 expression and type of TIM in ESCC patients. We found that PD-L1 expression and CD8+ T cell infiltration was not associated with the

clinicopathological features of ESCC. PD-L1 expression was an independent risk factor for the prognosis of ESCC. Immunotherapy may achieve promising outcomes in patients with type I TIM.

PD-L1 expression was studied limitedly in ESCC. Most reports evaluated the expression of PD-L1 by immunohistochemistry. Positive expression of PD-L1 was most commonly evaluated by the percentage of tumor cells with PD-L1 expression [21,22,28-30]. In the above mentioned five studies, positive rate of PD-L1 expression was 18.4%, 50.7%, 41.1%, 79.7% and 33.5%, respectively. Considering the positive rate of PD-L1 expression could not reflect the intensity of PD-L1 expression, IRS was employed in our present study. $IRS \geq 3$ was considered to be PD-L1 positive expression. As a result, the positive rate of PD-L1 expression was 61.7% in our present study. The differences of positive rate of PD-L1 expression may attribute to the different antibody of PD-L1 during immunohistochemistry, sample size and definition of PD-L1 expression.

The associations between clinicopathological features and PD-L1 expression were inconsistent in the previous reports. The majority of studies showed that none of the clinicopathological features was associated with PD-L1 expression [21,22,30,31]. However, Jiang et al. and Ito et al. reported that PD-L1 expression was associated with deeper tumor depth and lymph node metastasis [29,32]. Interestingly, Chen et al. found that PD-L1 expression was correlated with better tumor differentiation, lower third tumor, N0 stage and earlier tumor stage [28]. In our present study, we also did not find any association between clinicopathological features and PD-L1 expression. Findings about the associations between PD-L1 expression and prognosis of ESCC patients in the previous reports were controversial [19,22,28,29,32-38]. The majority of investigations reported that PD-L1 expression was associated with unfavorable prognosis of ESCC patients [19,29,32-35,37,38]. However, a few studies reported the opposite results [22,28,36]. Recently, a meta-analysis, including 8 studies with a number of 1350 ESCC patients, indicated that PD-L1 expression was correlated with poorer overall survival of ESCC patients [39]. In our present study, we also found that PD-L1 expression was an independent risk factor for unfavorable overall survival of ESCC patients.

CD8+ T cell plays an important role in the anti-tumor immunity [40], and the associations between CD8+ T cell infiltration and clinicopathological features of ESCC were investigated widely. The majority of studies showed that none of the clinicopathological features was associated with CD8+ T cell infiltration in ESCC

tissues [21,29,31,41]. However, two reports showed that CD8+ T cell infiltration was associated with male gender and elder patients, respectively [42,43]. Our present study observed a significant association between low density of CD8+ T cell infiltration and well differentiation status [44]. It has been reported that CD8+ T cell infiltration was correlated with favorable prognosis of ESCC [42,45]. However, other studies did not find any association between CD8+ T cell infiltration and prognosis of ESCC patients [21,29,43,46]. In our present study, we also found that CD8+ T cell infiltration was not associated with the prognosis of ESCC. The inconsistent results in different studies may attribute to race, sample size, and especially the components of infiltrating immune cells. It was reported that the prognosis of ESCC was also associated with the infiltration of regulatory T cells, neutrophils, tumor associated macrophages, dendritic cells and monocytes, etc. [21,42,43,47]. This may influence the results about prognostic value of CD8+ T cell infiltration in ESCC in the previous reports.

Type of TIM was reported to be associated with the prognosis of gastric cancer [48,49]. Ma et al. demonstrated that PD-L1-/CD8+ gastric cancer had the best overall survival, and PD-L1+/CD8- gastric cancer had the worst overall survival [48]. Koh et al. also showed that PD-L1-/CD8 high gastric cancer had the best overall survival, but PD-L1-/CD8 low gastric cancer had the worst overall survival [49]. Up to date, prognostic value of TIM by co-assessment of PD-L1 expression and CD8+ T cell infiltration has not been investigated in ESCC yet. Only two study showed that PD-L1-/CD3 low subgroup or PD-L1+ /TIM low subgroup associated with worse DFS and OS in ESCC patients, respectively [31,35]. Recently, a study showed that the high intratumoral CD3 infiltration was correlated with favourable OS and DFS. Moreover, type classification based on intratumoral CD3 infiltration and PD-L1 expression on tumor cell was an independent prognostic factor for nasopharyngeal carcinoma patients. Nevertheless, peritumoral CD3 infiltration showed non prognostic value for OS and DFS[50]. These observations revealed the importance of local complex interaction microenvironment. In present study, for the first time, we investigated the value of TIM classification based on intratumoral CD8+ T cell infiltration and PD-L1 expression on tumor cell, and found that PD-L1-/CD8 high had the best overall survival, whereas PD-L1+/CD8 high had the worst overall survival. PD-L1 expression on tumor cells could favorably prognoses clinical outcome in nasopharyngeal carcinoma patients with pre-existing intratumor CD3 infiltrating lymphocytes[50]. It was reported that

tumor regression after PD-L1/PD-1 blockade requires pre-existing CD8+ T cells [9]. In our study, we found that PD-L1+/CD8 high had the worst overall survival. From a treatment standpoint, this type of TIM maybe gain the maximum healing effect in potential immunotherapy of ESCC patients. Therefore, our findings indicated that type I (PD-L1+/CD8 high) ESCC could serve as candidate for anti-PD-1 or anti-PD-L1 immunotherapy.

There were some limitations in our present study. First, it was a retrospective study at a single institution, and the sample was relatively small. The findings in our study need further confirmation based on multicenter analysis with large sample size. Second, we did not analyze other tumor infiltrating immune cells in ESCC tissues. Thirdly, this study didn't investigate the relevance between the PD1 status on CD8+ T cells and clinical parameters. Fourthly, the disease free survival and disease specific survival of ESCC patients were not recorded and analyzed.

Conclusion

For the first time, the present study analyzed the associations between clinicopathological features and prognosis of ESCC patients and type of TIM based on co-assessment of PD-L1 expression and CD8+ T cell infiltration. We found that PD-L1 expression was not associated with clinicopathological features of ESCC. However, it was independently correlated with poor overall survival of ESCC. Further, we found that type of TIM was significantly associated with the prognosis of ESCC, which indicated that ESCC with type I TIM (PD-L1+/CD8 high) may achieve promising clinical outcomes through PD-L1/PD-1 blockade immunotherapy.

Abbreviations

PD-L1: programmed cell death ligand 1, ESCC: esophageal squamous cell carcinoma, TIM: tumor immune microenvironment.

Acknowledgments

This study was supported in part by grants from the National Natural Scientific Foundation of China [NO. 31570907, 81300301, 81572306, 81502403].

Authors' contributions

Qiao Wang, Fan Feng and Fei Wang contributed equally to this work. Hongwei Zhang and Fan Feng conceived and designed this study. Qiao Wang and Fei Wang carried on the immunohistochemistry staining. Qiao Wang wrote this manuscript. Gaozan Zheng, Fei Wang searched the literatures. Guanghui Xu, Zhen Liu, and Shushang Liu inputted the data.

Fan Feng, Fei Wang, Qiao Wang, Guanghui Xu, Zhen Liu, Shushang Liu, Man Guo, Xiao Lian analyzed the data.

Availability of data and materials

All relevant data of the study were already included in the manuscript.

Ethics approval and consent

This study was approved by the Ethics Committee of Xijing Hospital. Written informed consent was obtained from the patient for publication of this manuscript and any accompanying images.

Competing Interests

The authors have declared that no competing interest exists.

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