



Characterization of LAG-3, CTLA-4, and CD8⁺ TIL density and their joint influence on the prognosis of patients with esophageal squamous cell carcinoma

Wenjia Wang^{1#}, Donglai Chen^{2#}, Yuhuan Zhao^{1#}, Ting Zhao¹, Junmiao Wen³, Yiming Mao^{1,4}, Chang Chen², Yonghua Sang¹, Yongsheng Zhang⁵, Yongbing Chen¹

¹Department of Thoracic Surgery, The Second Affiliated Hospital of Soochow University, Suzhou 215004, China; ²Department of Thoracic Surgery, Shanghai Pulmonary Hospital, Tongji University, School of Medicine, Shanghai 200433, China; ³Department of Radiation Oncology, Fudan University Shanghai Cancer Center, Shanghai 200032, China; ⁴Department of Thoracic Surgery, Suzhou Kowloon Hospital Shanghai Jiaotong University School of Medicine, Suzhou 215028, China; ⁵Department of Pathology, The Second Affiliated Hospital of Soochow University, Suzhou 215004, China

Contributions: (I) Conception and design: W Wang, D Chen, Y Zhao; (II) Administrative support: Y Chen, Y Sang; (III) Provision of study materials or patients: Y Chen, Y Zhang; (IV) Collection and assembly of data: J Wen, Y Mao, T Zhao; (V) Data analysis and interpretation: Y Chen, C Chen; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Yongbing Chen. Department of Thoracic Surgery, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Gusu District, Suzhou 215004, China. Email: chentongt@sina.com; Yongsheng Zhang. Department of Pathology, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Gusu District, Suzhou 215004, China. Email: shengyongzh@126.com; Yonghua Sang. Department of Thoracic Surgery, The Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Gusu District, Suzhou 215004, China. Email: sangyh1983@sina.com.

Background: We aimed to characterize the relationships of lymphocyte activation gene-3 (LAG-3) expression, cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) expression, and CD8⁺ tumor-infiltrating lymphocyte (TIL) density, and to investigate the joint prognostic impact of these three markers in patients with surgically resected esophageal squamous cell carcinoma (ESCC).

Methods: Expression of LAG-3, CTLA-4 and the density of CD8⁺ TILs were evaluated by immunohistochemistry in resected ESCC. The associations between LAG-3 expression and clinicopathologic characteristics, as well as patient prognoses, were analyzed.

Results: A total of 183 patients were included. LAG-3 expression was observed in 69 (37.7%) patients. Positive LAG-3 expression was significantly associated with CTLA-4 expression ($P=0.004$). LAG-3 positivity, CTLA-4 positivity, and low CD8⁺ TIL densities were significantly associated with worsening recurrence-free survival (RFS) [LAG-3: hazard ratio (HR), 1.72; 95% confidence interval (CI), 1.10–2.89; $P=0.019$; CTLA-4: HR, 1.69; 95% CI, 1.04–2.73; $P=0.033$; CD8⁺: HR, 0.60; 95% CI, 0.38–0.94; $P=0.025$] and overall survival (OS) (LAG-3: HR, 2.09; 95% CI, 1.24–3.53; $P=0.006$; CTLA-4: HR, 1.47; 95% CI, 0.86–2.53; $P=0.161$; CD8⁺: HR, 0.56; 95% CI, 0.33–0.95; $P=0.032$). Subgroup analysis revealed that the LAG-3 CTLA-4 CD8⁺ group had the best RFS ($P<0.001$) and OS ($P<0.001$).

Conclusions: LAG-3 expression was correlated with CTLA-4 expression on TILs. Positive LAG-3 expression was associated with poor prognoses in ESCC. A combination of LAG-3, CTLA-4 expression and CD8⁺ TILs density could further stratify patients into different subgroups with distinct prognoses.

Keywords: Lymphocyte activation gene-3 (LAG-3); cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4); tumor-infiltrating lymphocytes (TIL); prognosis; esophageal squamous cell carcinoma (ESCC)

Submitted Aug 05, 2019. Accepted for publication Oct 22, 2019.

doi: 10.21037/atm.2019.11.38

View this article at: <http://dx.doi.org/10.21037/atm.2019.11.38>

Introduction

Esophageal cancer is a common malignant tumor of the digestive tract. Patients with esophageal squamous cell carcinoma (ESCC) (mainly in Eastern countries) suffer from high mortality rates, with a 5-year survival rate of less than 20% (1). At present, the treatment strategies for patients with ESCC mainly include cytotoxic chemotherapy, radiotherapy, and surgery. These modalities convey small survival benefits and significant adverse effects on patients (2,3). Therefore, finding novel and more effective therapeutic strategies for patients with ESCC remains an urgent need.

The last decade has witnessed the rapid development of immunotherapy, which can reverse tumor immune escape mechanisms by suppressing immune checkpoints (4). The inhibition of cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) can activate T cells to eliminate tumors (5,6). The inhibitors of CTLA-4 have shown promising efficacy in a variety of cancers (7,8). However, the efficacy of anti-CTLA-4 antibodies is still unproven in ESCC. Moreover, less than 20% of patients are expected to benefit from anti-CTLA-4 therapy (9), which highlights the necessity for further investigation into other checkpoint modulators in patients with ESCC, especially those associated with adaptive resistance to CTLA-4 inhibition.

Lymphocyte activation gene-3 (LAG-3) is another important immune checkpoint which belongs to the immunoglobulin superfamily. LAG-3 is expressed in various kinds of immune cells, including tumor-infiltrating lymphocytes (TILs), which play a key role in inhibiting T cell proliferation, activation, and homeostasis (10,11). LAG-3 is not only a surface molecule selectively upregulated on regulatory T (Treg) cells but also a key modulator of maximal Treg activity (12,13). LAG-3 can downregulate CD4⁺ T cell activity through binding with the major histocompatibility complex II (MHC class II) (14,15). Similarly, CTLA-4 can enhance Treg immunosuppressive activity and downregulate CD4⁺ T cell activity, which may have a synergistic effect with LAG-3 (6,12,16). It has been reported that LAG-3 expression on TILs was significantly correlated with that of programmed death 1 (PD-1) on TILs and programmed death ligand 1 (PD-L1) on tumor cells (17). However, the relationship between LAG-3 and CTLA-4 on TILs has not been investigated. LAG-3 has been demonstrated to be a favorable prognostic factor in ESCC (18,19) in contrast to its negative prognostic impact in other cancers, including head and neck squamous cell carcinoma, gastric

cancer, and lung cancer (17,20,21). In order to promote and optimize the future application of immunotherapy in operable ESCC, we aimed to comprehensively explore the clinicopathological features of LAG-3 expression and the association between positive LAG-3 expression and clinical outcomes in patients with ESCC after surgical resection. More importantly, we also assessed the relationships between LAG-3, CTLA-4 expression, and CD8⁺ TIL density and further investigated the prognostic value of the different combinations of these three markers.

Methods

Patients

We retrospectively reviewed 261 patients with esophageal cancer who underwent surgical treatment in our department in the Second Affiliated Hospital of Soochow University, China, from January 2009 to December 2014. The patients in our study had to meet the following inclusion criteria: (I) patients that had not undergone any systemic therapy before surgery, and (II) patients who were pathologically confirmed with primary squamous cell carcinoma. The exclusion criteria were (I) patients with autoimmune diseases and other kinds of esophageal cancer (e.g., adenocarcinoma), (II) patients lost to follow-up, and (III) patients with concurrent multiple primary tumors or other malignancies. According to the criteria, 78 patients were excluded, while 183 patients were included in the current study. This study was approved by the Institutional Review Board of the Second Affiliated Hospital of Soochow University. Because of the retrospective nature of this study, the informed consent of patients was waived.

Immunohistochemistry

Three serial 4- μ m-thick formalin-fixed paraffin-embedded tissue sections were taken from the same tumor specimen for LAG-3, CTLA-4, and CD8⁺ staining. First, the sections were deparaffinized and rehydrated, and endogenous peroxidase was quenched with 10% H₂O₂ at room temperature for 10 min. After this, nonspecific proteins were blocked with 10% goat serum for 1 h. The sections were then rinsed and incubated with the detection antibody [LAG-3 (Abcam, ab40465, China), CTLA-4 (Biorbyt, orb385624, China) and CD8⁺ (Abcam, ab4055, China)] overnight at 4 °C. The DAB Horseradish Peroxidase Color Development Kit (Beyotime, China) was used for color

development after incubating with horseradish peroxidase conjugated secondary antibody at room temperature for 30 min. Cell nuclei were counterstained with hematoxylin, and the slides were dehydrated in an ethanol gradient, mounted with neutral gum, and stored for downstream analyses.

Immunohistochemistry evaluation

All specimens were determined by two experienced independent pathologists who were blind to the data (Li F and Zhang Y). Five visual fields were randomly selected in each section. Cells were regarded as positive if the cell membrane and/or cytoplasm stained brown. The intensity of staining and the percentage of positive cells were evaluated in all sections, and the final score was derived from the multiplication of the two parameters. The scoring system for staining intensity was as follows: one point, absent/weak staining; two points, moderate staining; three points, strong staining. The scoring system for the percentage of positive cells was as follows: one point, $\leq 33\%$; two points, $>33\%$ to 66% ; three points, $>66\%$. Sections with a final overall score of ≤ 3 were defined as the LAG-3 or CTLA-4 negative expression group; other sections were defined as the high expression group (22,23). CD8⁺ TIL density was evaluated both in the tumor parenchyma and mesenchymal area and was defined as low CD8⁺ TIL density if infiltration was $<1\%$ in the parenchyma and $<10\%$ in the mesenchyme at the same time, and defined as high CD8⁺ TILs density for other values (24). A consensus was reached after discussion if there were controversies or discordance in terms of immunohistochemical evaluation.

Statistical analyses

All clinical data are shown as mean \pm standard deviation and n (%). We performed the chi-square test to assess the correlation between LAG-3 and clinical pathological variables. Spearman's rank correlation analysis was used to analyze the association between LAG-3 and CTLA-4 or CD8⁺. We used variables of $P < 0.2$ for a logistic regression model to investigate the independent predictive factors of LAG-3 expression. Also, the log-rank test was used to compare the survival for distinct groups of a variable. To evaluate the independent predictive factors for recurrence free survival (RFS) and overall survival (OS), a Cox proportional hazard regression model was applied to evaluate the prognostic impact of a potentially survival-

related variable. The survival curves were estimated by the Kaplan-Meier method. Statistical analyses were performed in SPSS 25.0 (IBM Corporation, Armonk, NY, USA). In addition, we used the Tumor IMMune Estimation Resource (TIMER, a website based on the Cancer Genome Atlas database, <https://cistrome.shinyapps.io/timer/>) to explore differential gene expression in tumors and normal tissues as well as the correlation between LAG-3 and CTLA-4, which served as external validation. In our study, a two-sided P value of less than 0.05 was considered statistically significant.

Results

Baseline information

The clinical characteristics of the 183 patients are shown in *Table 1*. The mean age at diagnosis was 63 years (range, 35–81 years). Patients were followed up until their death or last follow-up (median: 56 months). In our cohort, 147 patients (80.3%) were male, and 36 (19.7%) were female. Seventy-three patients (39.9%) were non-smokers. Eight (4.4%), 115 (62.8%), and 60 (32.8%) patients had upper, middle, and lower thoracic ESCC, respectively.

Associations between LAG-3 expression and clinicopathological characteristics

According to the chi-square test, no statistically significant association was found between LAG-3 expression and age, sex, smoking status, tumor location, T stage, N stage, pathological differentiation, vascular invasion, perineural involvement, or surgical type (*Table 1*). However, the results of Spearman's rank correlation analysis showed that LAG-3 positivity was significantly associated with N stage ($r=0.154$, $P=0.038$) (*Table S1*).

Associations between LAG-3, CTLA-4 and CD8⁺ expression

As shown in *Figure 1*, LAG-3, CTLA-4, and CD8⁺ were expressed on TILs but were not found on tumor cells. Positive LAG-3, CTLA-4, and CD8⁺ expression was detected in 69 (37.7%), 86 (47.0%), and 88 (48.1%) patients, respectively. LAG-3 positivity was significantly associated with positive CTLA-4 expression ($P < 0.001$) and high CD8⁺ TIL density ($P=0.013$, *Table 1*). Spearman's rank correlation analyses also demonstrated the same results

Table 1 Correlation between LAG-3 expression and clinicopathologic parameters

Variables	LAG-3 expression		P
	Negative (N=114)	Positive (N=69)	
Age (years), mean \pm SD	62.1 \pm 9.5	64.7 \pm 8.4	0.058
\leq 65	70 [61]	36 [52]	0.220
>65	44 [39]	33 [48]	
Sex, n [%]			0.870
Male	92 [81]	55 [80]	
Female	22 [19]	14 [20]	
Smoking, n [%]			0.646
Non-smoker	44 [39]	29 [42]	
Smoker	70 [61]	40 [58]	
Tumor location, n [%]			0.180
Upper	6 [5]	2 [3]	
Middle	76 [67]	39 [57]	
Lower	32 [28]	28 [41]	
T stage, n [%]			0.635
T1	6 [5]	5 [7]	
T2	43 [38]	31 [45]	
T3	57 [50]	28 [41]	
T4	8 [7]	5 [7]	
N stage, n [%]			0.051
N0	80 [70]	37 [54]	
N1	27 [24]	28 [41]	
N2	7 [6]	4 [6]	
Pathologic differentiation, n [%]			0.170
High	10 [9]	2 [3]	
Moderate	79 [69]	46 [67]	
Poor	25 [22]	21 [30]	
Vascular invasion, n [%]			0.982
Absent	99 [87]	60 [87]	
Present	15 [13]	9 [13]	
Perineural involvement, n [%]			0.360
Absent	101 [89]	64 [93]	
Present	13 [11]	5 [7]	

Table 1 (continued)

Table 1 (continued)

Variables	LAG-3 expression		P
	Negative (N=114)	Positive (N=69)	
Surgical type, n [%]			0.212
Sweet	39 [34]	27 [39]	
Ivor-Lewis	42 [37]	30 [43]	
McKeown	33 [29]	12 [17]	
CTLA-4 expression, n [%]			<0.001
Negative	72 [63]	25 [36]	
Positive	42 [37]	44 [64]	
CD8 expression, n [%]			0.013
Negative	51 [45]	44 [64]	
Positive	63 [55]	25 [36]	

LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; TIL, tumor-infiltrating lymphocyte.

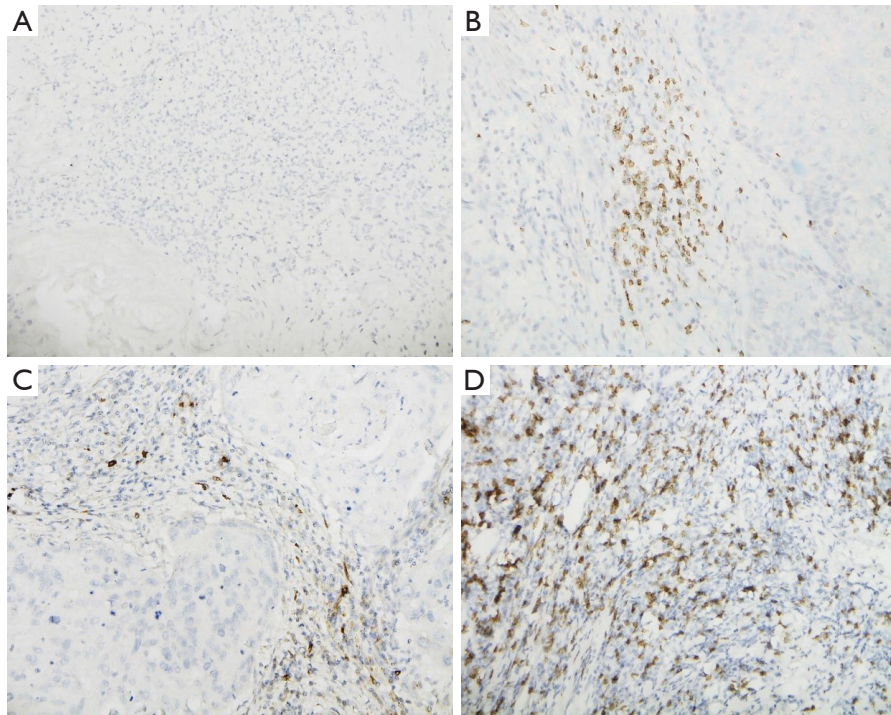


Figure 1 LAG-3 negative expression on TILs and positive IHC staining for LAG-3, CTLA-4, and CD8*. (A) LAG-3 negative expression on TILs; (B) IHC positivity for LAG-3; (C) IHC positivity for CTLA-4; (D) IHC positivity for CD8*. (Photograph magnification: $\times 400$). TILs, tumor-infiltrating lymphocytes; IHC, immunochemistry; LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

Table 2 Multivariate logistic regression model for LAG-3 expression in patients with esophageal squamous cell carcinoma

Variables	Multivariate	
	OR (95% CI)	P
Tumor location (upper vs. middle & lower)	0.60 (0.11–3.34)	0.559
N stage (N0 vs. N1-2)	0.55 (0.29–1.06)	0.076
Pathologic differentiation (high vs. moderate & poor)	0.24 (0.05–1.25)	0.091
CTLA-4 expression (negative vs. positive)	0.38 (0.20–0.74)	0.004
CD8 expression (negative vs. positive)	1.81 (0.94–3.47)	0.075

LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; TIL, tumor-infiltrating lymphocyte; OR, odds, ratio.

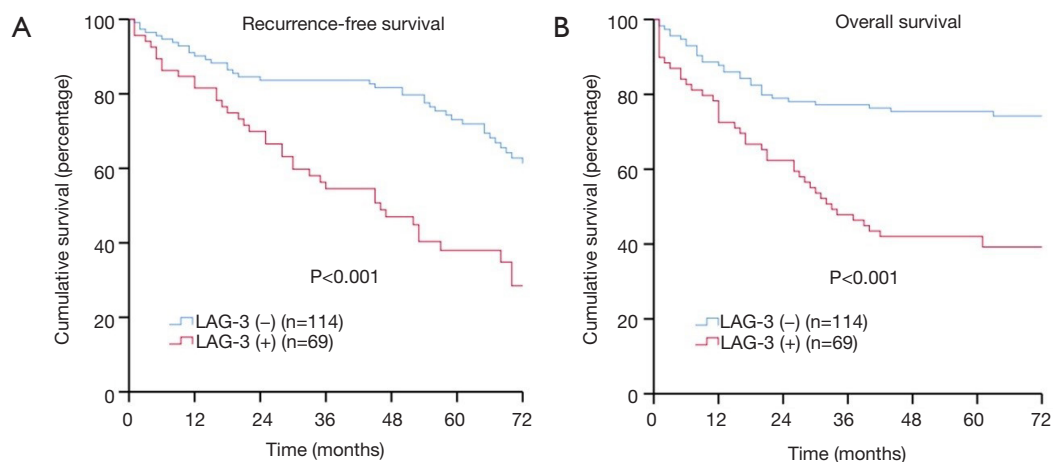


Figure 2 LAG-3 positivity, recurrence-free survival (RFS), and overall survival (OS) in patients with esophageal squamous cell carcinoma (ESCC). (A) RFS by LAG-3; (B) OS by LAG-3. LAG-3, lymphocyte activation gene-3.

(LAG-3 and CTLA-4: $r=0.261$, $P<0.001$; LAG-3 and $CD8^+$: $r=-0.185$, $P=0.012$) (Table S1). Moreover, further multivariate logistic regression analysis indicated that only CTLA-4 positivity [odds ratio (OR), 0.38; 95% CI, 0.20–0.74; $P=0.004$] was an independent predictive factor for LAG-3 expression (Table 2).

Prognostic value of LAG-3, CTLA-4 and $CD8^+$ expression

As shown in Figure 2A,B, the log-rank tests revealed that patients with negative LAG-3 expression had significantly better RFS (5-year rate: 58.8% versus 40.6%, $P<0.001$) and OS (5-year rate: 74.6% versus 42.0%, $P<0.001$) compared with those with positive LAG-3 expression. Meanwhile, patients with CTLA-4 negative expression had significantly better survival compared to those with positive CTLA-4 expression (5-year RFS rate: 60.8% versus 43.0%,

$P<0.001$; 5-year OS rate: 74.2% versus 47.7%, $P<0.001$) (Figure S1A,B). Additionally, patients with low $CD8^+$ TIL density had significantly lower survival when compared to those with high $CD8^+$ TIL density (5-year RFS rate: 44.2% versus 61.4%, $P=0.002$; 5-year OS rate: 51.6% versus 72.7%, $P=0.001$) (Figure S1C,D).

Correlation between a combination of LAG-3 and CTLA-4 expression and/or $CD8^+$ TIL density, and clinical outcomes

Co-expression of LAG-3, CTLA-4, and $CD8^+$ was present in different combinations in our patient group. We had (I) 44 patients with LAG-3⁺ CTLA-4⁺, (II) 25 patients with LAG-3⁺ $CD8^+$, (III) 31 patients with CTLA-4⁺ $CD8^+$, (IV) 10 patients with LAG-3⁺ CTLA-4⁺ $CD8^+$ (Figure S2). The combination of LAG-3 and/or CTLA-4 expression or $CD8^+$ TIL density could be used to further stratify patients

into subgroups with distinct prognoses. Patients with LAG-3⁻ CTLA-4⁻ had the best prognoses, patients with LAG-3⁻ CTLA-4⁻ or LAG-3⁻ CTLA-4⁺ had moderate prognoses, and patients with LAG-3⁺ CTLA-4⁺ had the worst prognoses (RFS: $P < 0.001$; OS: $P < 0.001$) (Figure 3A,B). Patients with LAG-3⁻/CTLA-4⁻ CD8⁺ had the best prognoses, patients with LAG-3⁺/CTLA-4⁺ CD8⁺ or LAG-3⁻/CTLA-4⁻ CD8⁻ had moderate prognoses, and patients with LAG-3⁺/CTLA-4⁺ CD8⁻ had the worst prognoses (RFS: $P < 0.001/P < 0.001$; OS: $P < 0.001/P < 0.001$) (Figure 3C,D,E,F). Moreover, patients with LAG-3⁻ CTLA-4⁻ CD8⁺ had the best prognoses, and patients with LAG-3⁺ CTLA-4⁺ CD8⁻ had the worst prognoses (RFS: $P < 0.001$; OS: $P < 0.001$) (Figure 4A,B).

Multivariate analysis of RFS and OS

We included the variables of $P < 0.2$ [RFS: age ($P = 0.164$), N stage ($P < 0.001$), LAG-3 expression ($P < 0.001$), CTLA-4 expression ($P < 0.001$), and CD8⁺ expression ($P = 0.002$); OS: age ($P = 0.153$), N stage ($P < 0.001$), perineural involvement ($P = 0.141$), LAG-3 expression ($P < 0.001$), CTLA-4 expression ($P < 0.001$), and CD8⁺ expression ($P = 0.001$)] in the univariate analyses and survival-associated variables into the Cox regression analyses. As shown in Table 3, regional lymph node metastasis [hazard ratio (HR), 1.88; 95% CI, 1.20–2.94; $P = 0.006$], LAG-3 positivity (HR, 1.72; 95% CI, 1.10–2.89; $P = 0.019$) and CTLA-4 positivity (HR, 1.69; 95% CI, 1.04–2.73; $P = 0.033$) were independent prognostic factors of worsening RFS. Conversely, high CD8⁺ TIL density (HR, 0.60; 95% CI, 0.38–0.94; $P = 0.025$) was a favorable indicator of superior RFS. Moreover, regional lymph node metastasis (HR, 1.97; 95% CI, 1.20–3.23; $P = 0.007$) and LAG-3 positivity (HR, 2.09; 95% CI, 1.24–3.53; $P = 0.006$) were independent risk factors of worsening OS, whereas high CD8⁺ TIL density (HR, 0.56; 95% CI, 0.33–0.95; $P = 0.032$) represented a favorable predictor for better OS.

Discussion

As shown using TIMER, the respective expression level of LAG-3 and CTLA-4 in tumor tissues was significantly higher than that in normal tissues (LAG-3: $P < 0.05$; CTLA-4: $P < 0.001$) in patients with esophageal cancer (Figures S3,S4). Furthermore, LAG-3 expression levels were significantly correlated with CTLA-4 expression levels in tumor tissues ($r = 0.781$; $P < 0.001$) (Figure S5), which partly

supported our results. To the best of our knowledge, this is the first study to characterize LAG-3 and CTLA-4 expression, along with their prognostic significance in patients with surgically resected ESCC. We found that CTLA-4 positivity was an independent predictive factor for LAG-3 expression. Moreover, positive LAG-3 expression, positive CTLA-4 expression, and low CD8⁺ TIL density were independent predictors of worsening RFS and OS. The combination of these three markers could further stratify patients into different subgroups with distinct prognoses.

The use of immunotherapy is a milestone for cancer treatment, which activates the immune system to eradicate tumor cells. However, the exact clinical utility of immunotherapy for ESCC is still unclear. LAG-3 is a promising target because it is one of the key inhibitory receptors that contributes to T-cell exhaustion (25). Anti-LAG-3 inhibitors can activate T effector cells and reduce the immunosuppressive activity of Tregs, whereas anti-PD-1 and anti-PD-L1 inhibitors do not reduce the activity of Tregs (26). Furthermore, studies have reported that LAG-3 plays an important role in the immune response and the relative safety of anti-LAG-3 inhibitors in animal models (27).

Additionally, LAG-3 inhibitors (such as Relatlimab, IMP321, BMS-986016, LAG525, MK-4280, TSR-032) have entered clinical trials for different cancers, either alone or in combination, and are showing promising efficacy (28–30). It was reported that the blockade of LAG-3 and other checkpoints could synergistically enhance T-cell activity and anti-tumor immunity (31). However, there has been no study characterizing the relationships between LAG-3 and CTLA-4. Since LAG-3 and CTLA-4 have similar effects in suppressing the immune system by down-regulating CD4⁺ T cells and enhancing Tregs, blockade of LAG-3 may help overcome the adaptive resistance of CTLA-4 inhibitors, which could also be helpful in improving CTLA-4 inhibition-based therapy. Therefore, anti-LAG-3 inhibitors may exhibit greater efficacy in the future immunotherapy of ESCC patients with positive LAG-3 expression (32).

Two previous studies have demonstrated that LAG-3 was a favorable prognostic factor for patients with ESCC (18,19), which conflicts with our results. The potential reason for this discrepancy may be attributed to the following: (I) different analytical methods and (II) different definitions of positive LAG-3 expression. Specifically, expression levels based on immunohistochemical staining may be different from those measured by mRNA microarrays. Also, an

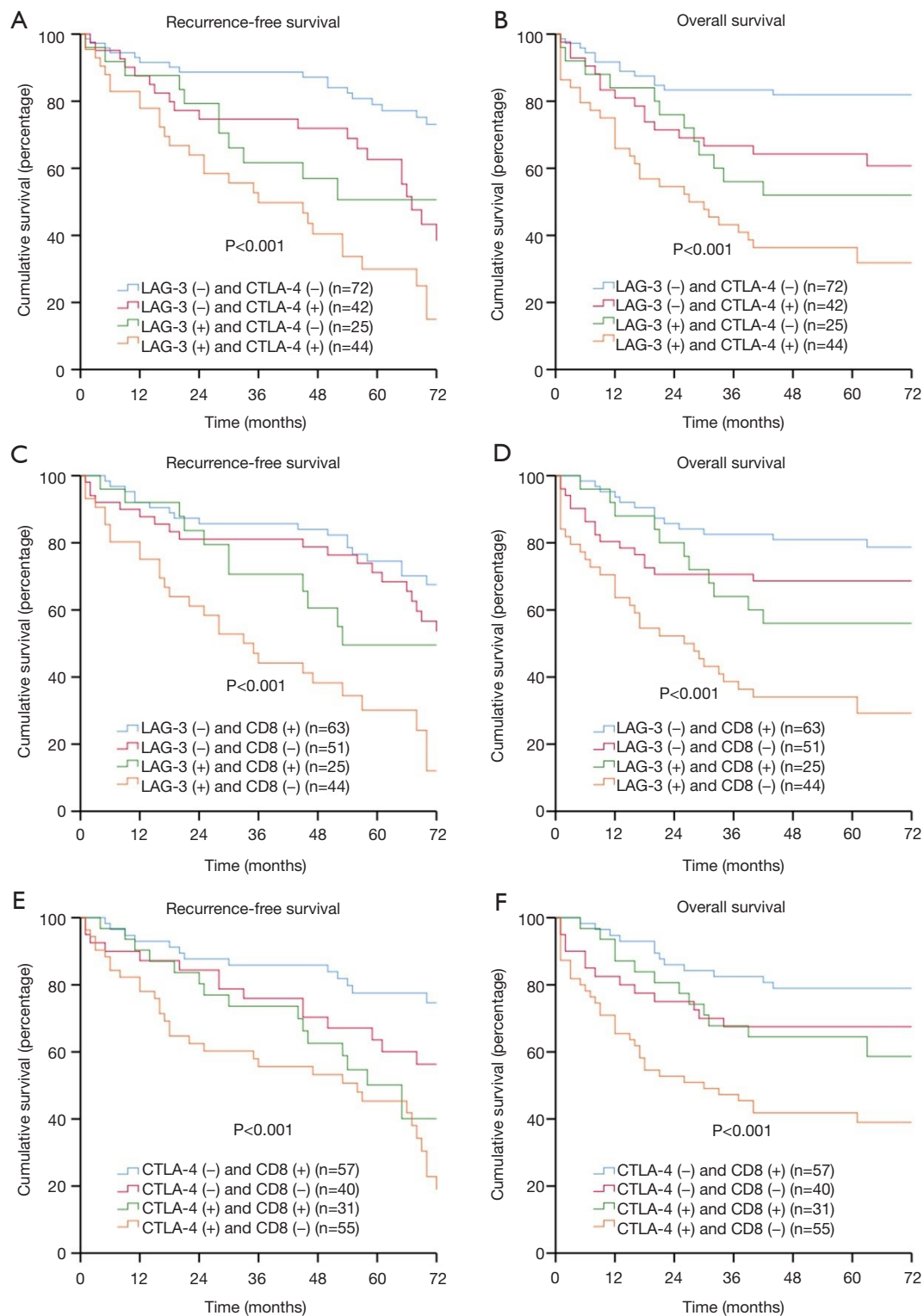


Figure 3 LAG-3, CTLA-4, CD8⁺, recurrence-free survival (RFS), and overall survival (OS) in patients with esophageal squamous cell carcinoma (ESCC). (A) RFS by LAG-3 and CTLA-4; (B) OS by LAG-3 and CTLA-4; (C) RFS by LAG-3 and CD8⁺; (D) OS by LAG-3 and CD8⁺; (E) RFS by CTLA-4 and CD8⁺. (F) OS by CTLA-4 and CD8⁺. LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

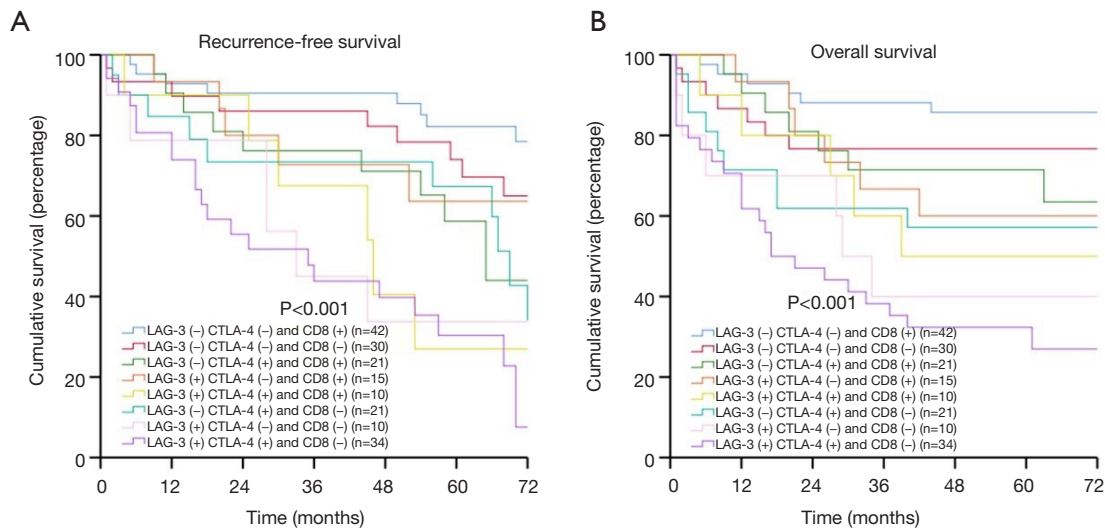


Figure 4 LAG-3, CTLA-4, CD8⁺, and recurrence-free survival (RFS), overall survival (OS) in patients with esophageal squamous cell carcinoma ESCC. (A) RFS by LAG-3, CTLA-4, and CD8⁺; (B) OS by LAG-3, CTLA-4, and CD8⁺. LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

Table 3 Cox proportional-hazards regression model for recurrence-free survival (RFS) and overall survival (OS) in all patients

Variables	Recurrence-free survival			Overall survival		
	Univariate	Multivariate		Univariate	Multivariate	
	P	HR (95% CI)	P	P	HR (95% CI)	P
Age (> 65 vs. ≤65)	0.164	1.12 (0.72–1.75)	0.625	0.153	1.24 (0.76–2.03)	0.386
Sex (female vs. male)	0.724			0.844		
Smoking (current or ex vs. non-smoker)	0.243			0.240		
Tumor location (middle & lower vs. upper)	0.959	0.93 (0.29–3.01)	0.903	0.411	2.31 (0.48–11.20)	0.299
T stage (T2-4 vs. T1)	0.255	2.34 (0.67–8.12)	0.181	0.396	1.48 (0.43–5.13)	0.538
N stage (N1-2 vs. N0)	<0.001	1.88 (1.20–2.94)	0.006	<0.001	1.97 (1.20–3.23)	0.007
Pathologic differentiation (moderate & poor vs. high)	0.724	0.94 (0.36–2.44)	0.892	0.377	1.22 (0.37–4.04)	0.746
Vascular invasion (present vs. absent)	0.401			0.282		
Perineural involvement (present vs. absent)	0.624			0.141	0.42 (0.15–1.19)	0.102
Surgical type (McKeown vs. Sweet & Ivor-Lewis)	0.485	1.20 (0.71–2.05)	0.493	0.387	1.55 (0.88–2.71)	0.128
LAG-3 (positive vs. negative)	<0.001	1.72 (1.10–2.89)	0.019	<0.001	2.09 (1.24–3.53)	0.006
CTLA-4 (positive vs. negative)	<0.001	1.69 (1.04–2.73)	0.033	<0.001	1.47 (0.86–2.53)	0.161
CD8 (positive vs. negative)	0.002	0.60 (0.38–0.94)	0.025	0.001	0.56 (0.33–0.95)	0.032

Variables with P value <0.2 in univariate models and variables clinically considered to have an impact on survival were analyzed in a multivariate analysis model. LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; TIL, tumor-infiltrating lymphocyte; HR, hazard ratio.

optional cut-off value for defining high and low LAG-3 expression may affect the survival analysis of the patients. More importantly, the two studies mentioned above demonstrated that the expression of LAG-3 in tumor tissues was significantly higher than that in surrounding tissues, and blocking LAG-3 could activate CTLs, suggesting that LAG-3 may be involved in tumorigenesis, and therefore, the blockade of LAG-3 may activate the immune system.

As shown in our study, positive LAG-3 expression significantly correlated with positive CTLA-4 expression. The combination of different expression levels among LAG-3, CTLA-4, and CD8⁺ could stratify patients into more detailed subgroups. According to the Kaplan-Meier curves for RFS and OS, the expression of a single immune checkpoint is not enough to determine the use of an immune inhibitor. For instance, when CTLA-4 and CD8⁺ expression was positive, the prognosis was not affected regardless of LAG-3 expression. Therefore, when positive LAG-3 expression was detected alone, substantial survival benefit would not be obtained in patients treated with anti-LAG-3 inhibitors alone. More importantly, for ESCC expressing both LAG-3 and CTLA-4, combined immunotherapy targeting LAG-3 and CTLA-4 could be more effective, especially for those infiltrated with abundant CD8⁺ T cells. This is because the cytotoxicity of pre-existing T cells would be turned off by LAG-3 and CTLA-4 engagement. Taken together, for current immunotherapy, the expression levels of multiple immune checkpoints should be monitored. A combination of LAG-3 inhibitors and CTLA-4 inhibitors may be an effective method for patients with ESCC. Additionally, the combination of LAG-3 and PD-1/PD-L1 may be a future direction of more attempts.

There are some limitations to our study. First, because of the retrospective nature of this study, selection bias and performance bias were inevitable. Second, the number of patients enrolled in our study was limited, which potentially affected our subgroup analyses, as patients with esophageal adenocarcinoma were not included in our study. Further prospective multicenter studies are warranted to address these limitations.

Conclusions

In summary, LAG-3 is expressed on TILs in patients with ESCC. Positive LAG-3 expression was significantly associated with positive CTLA-4 expression and poor

prognosis in ESCC. LAG-3, CTLA-4 expression, and CD8⁺ TIL density were independent prognostic factors for clinical outcomes of ESCC patients. Furthermore, we found that diverse prognostic features were exhibited among subgroups stratified by the expression levels of LAG-3, CTLA-4, and CD8⁺. The combined expression of LAG-3, CTLA-4, and CD8⁺ in ESCC may serve as predictive biomarkers for immunotherapy in the future.

Acknowledgments

We want to thank Dr. Feng Li for his substantial aid in the review of the paraffin sections.

Funding: This work was supported by the Suzhou Key Discipline for Medicine (SZXK201803), the Suzhou Key Laboratory of Thoracic Oncology (SZS201907), the Municipal Program of People's Livelihood Science and Technology in Suzhou (SS2019061), and the Municipal Program of Science and Technology in Suzhou (SYS201725).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Institutional Review Board of the Second Affiliated Hospital of Soochow University.

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Cite this article as: Wang W, Chen D, Zhao Y, Zhao T, Wen J, Mao Y, Chen C, Sang Y, Zhang Y, Chen Y. Characterization of LAG-3, CTLA-4, and CD8⁺ TIL density and their joint influence on the prognosis of patients with esophageal squamous cell carcinoma. *Ann Transl Med* 2019;7(23):776. doi: 10.21037/atm.2019.11.38

Table S1 Correlation between LAG-3 expression and clinicopathological parameters

Variables	Correlation with LAG-3, r	P
Age	0.091	0.233
Sex	0.012	0.871
Smoking	-0.034	0.648
Tumor location	0.134	0.070
T stage	-0.081	0.277
N stage	0.154	0.038
Pathologic differentiation	0.127	0.087
Vascular invasion	-0.002	0.982
Perineural involvement	-0.068	0.363
Surgical type	-0.099	0.180
CTLA-4	0.261	<0.001
CD8	-0.185	0.012

LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4; TIL, tumor-infiltrating lymphocyte.

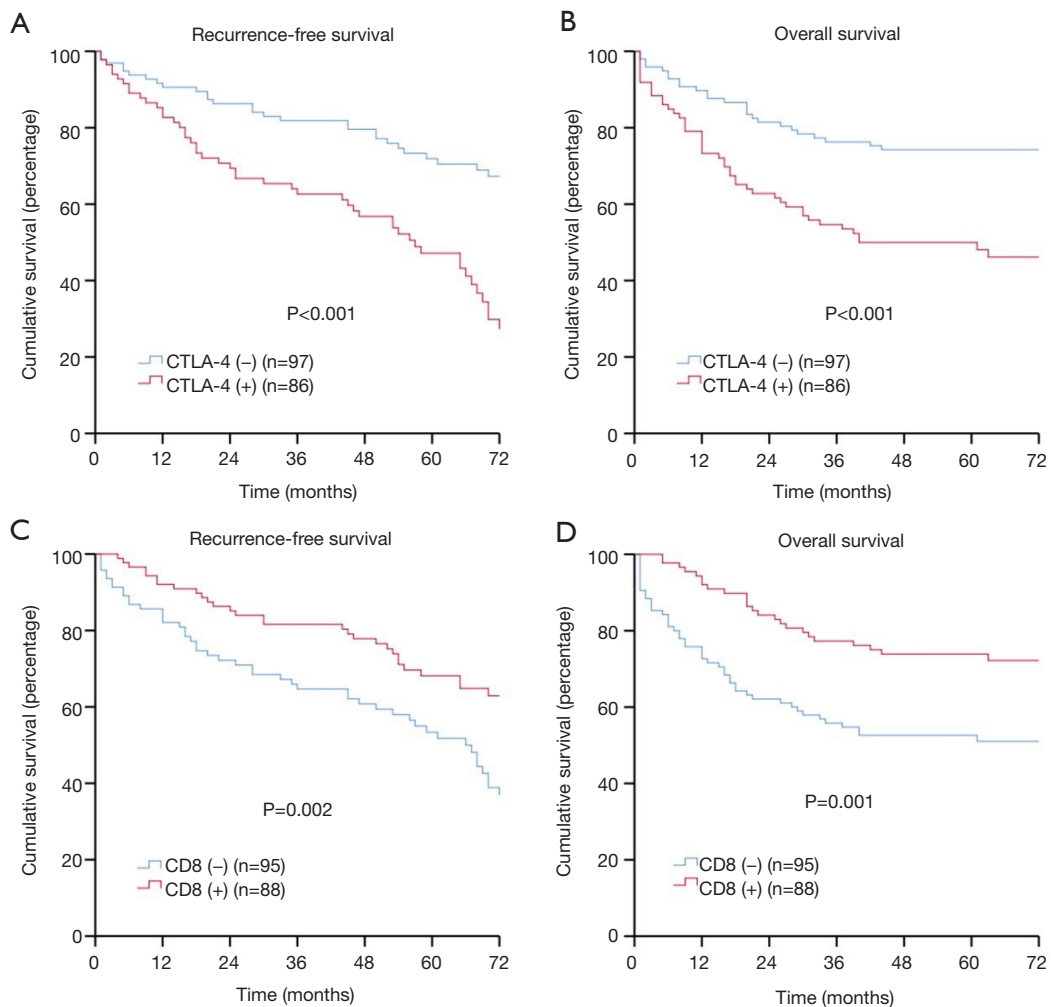


Figure S1 CTLA-4 positivity, CD8⁺, and recurrence-free survival (RFS), overall survival (OS) in patients with esophageal squamous cell carcinoma (ESCC). (A) RFS by LAG-3; (B) OS by LAG-3; (C) RFS by CD8⁺; (D) OS by CD8⁺. CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

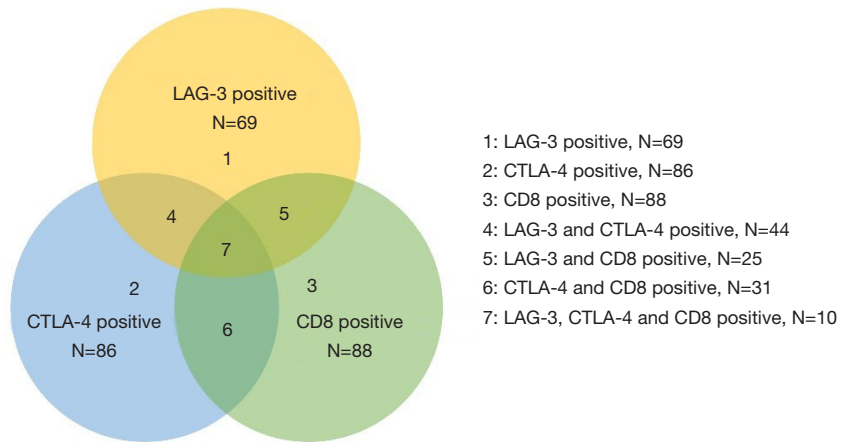


Figure S2 Description of LAG-3, CTLA-4, CD8+ expression, and co-expression. LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

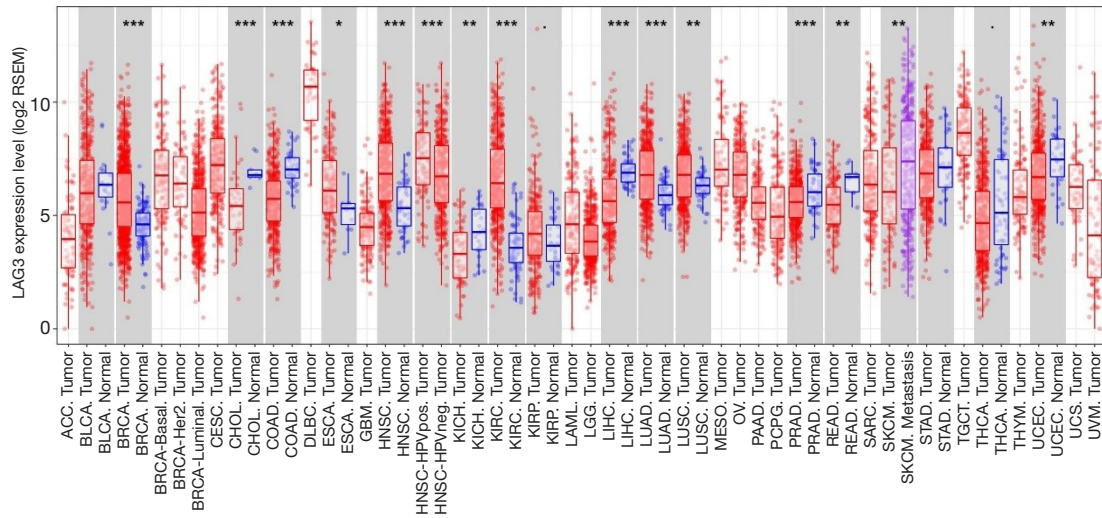


Figure S3 The expression levels of LAG-3 in different cancers. LAG-3, lymphocyte activation gene-3.

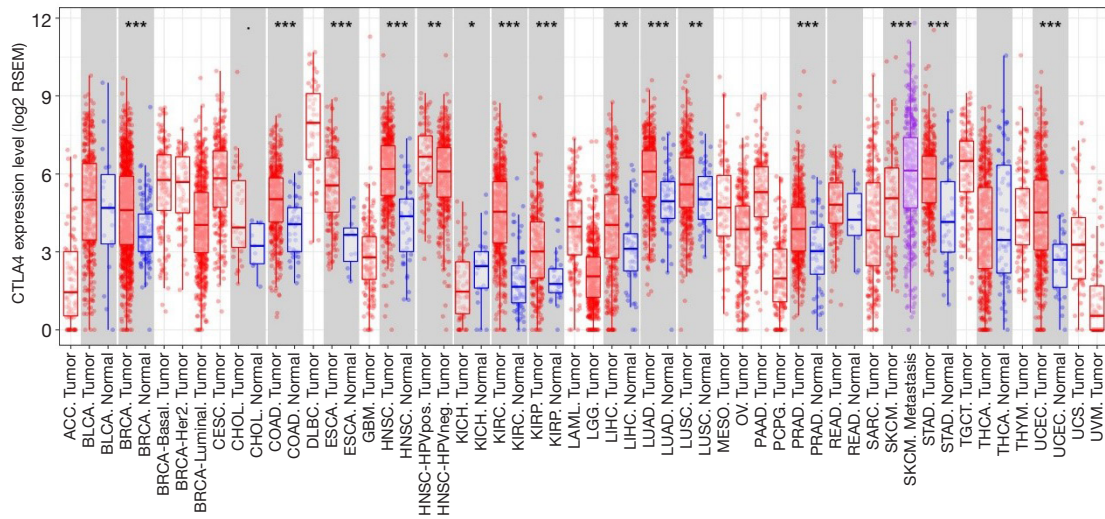


Figure S4 The expression levels of CTLA-4 in different cancers. CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.

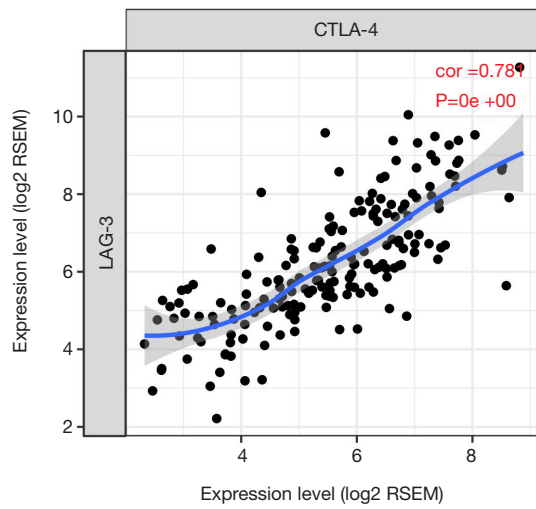


Figure S5 Correlation between LAG-3 expression levels and CTLA-4 expression levels in patients with esophageal cancer. LAG-3, lymphocyte activation gene-3; CTLA-4, cytotoxic T-lymphocyte-associated antigen-4.