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ORIGINAL ARTICLE

Tumor-Infiltrating CD1a$^+$ DCs and CD8$^+$/FoxP3$^+$ Ratios Served as Predictors for Clinical Outcomes in Tongue Squamous Cell Carcinoma Patients

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Abstract

Tumor-infiltrating immune cells engage in an extensive crosstalk with tumors and act as two-edged swords by inhibiting or promoting cancer growth. Therefore, identifying the density and prognostic values of tumor-infiltrating immune cells will provide valuable tips for cancer treatments. In this study, we identified the density of tumor inflammatory infiltrates and the number of tumor-infiltrating immune cells, including CD3$^+$, CD4$^+$, CD8$^+$, FoxP3$^+$ T cells and CD1a$^+$ dendritic cells (DCs) in 153 tongue squamous cell carcinomas (TSCC). High inflammatory cell infiltration was associated with better overall survival (OS) and disease free survival (DFS). Moreover, the number of CD3$^+$, CD4$^+$, FoxP3$^+$ and CD1a$^+$ cells were associated with tumor differentiation ($P<0.001$) and the number of FoxP3$^+$, CD1a$^+$ cells and CD8$^+$/FoxP3$^+$ ratios were also associated with tumor stage ($P<0.01$, $P<0.01$, $P<0.05$). In addition, patients with higher CD1a$^+$ DCs had better OS and increased CD8$^+$/FoxP3$^+$ ratios were associated with improved OS and DFS ($P=0.037$; $P=0.047$; $P=0.033$). In conclusion, our results indicated that tumor-infiltrating CD1a$^+$ DCs and CD8$^+$/FoxP3$^+$ ratios were associated with favorable clinical outcomes but not independent prognostic factors for TSCC patients.

Keywords Tongue squamous cell carcinoma · Tumor infiltrating immune cell · Clinical outcome · Diagnostic value

Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide and five-year survival rate of OSCC remains 50% for 10 years, posing a significant health threat. OSCC arises from oral tongue, floor of mouth, buccal surface, alveolar surface and hard palate, but the most common subsite is the tongue, which is increasing in incidence and has an aggressive clinical behavior with poor prognosis [1]. Therefore, it is important to identify valuable biomarkers that accurately predict the behavior of OSCC and help the selection of appropriate treatments.

The immune system interacts with cancer cells through a complex system, and plays a dual role in inhibiting or promoting tumor growth [2]. Therefore, tumor-infiltrating immune cells, which accurately reflect host immune interaction, were studied as biomarkers in different tumors. For example, CD8$^+$ T lymphocytes are associated with favorable outcomes in a broad range of tumor types, such as breast cancer, ovarian cancer, head and neck cancer and lung cancer et al. [3–6]. In contrast, other immune cells, such as myeloid-derived suppressor cells and regulatory T cells (Tregs), appear to have an adverse role in the immune control of cancers and are therefore associated with worse outcomes [7–9]. However, no unambiguous conclusions can be drawn from studies on

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the prognostic value of tumor-infiltrating cells in OSCC as conflicting results were reported [5, 10–14]. Increasing evidence suggested that the density and prognostic values of tumor-infiltrating cells were various in different anatomical subsites [10]. For example, $FoxP3^+$ cells were associated with worse clinical outcome in head and neck cancers, while the opposite correlation was seen in tonsillar tumors [11, 15, 16]. Therefore, a more homogeneous cohort of patients is required.

Although previous studies have examined the prognostic values of tumor-infiltrating cells, they have limitations of small sample size, limited immune cell types or a heterogeneous cohort of patients. Therefore, in this study 153 tongue squamous cell carcinoma (TSCC) patients were enrolled to clarify the number and diagnostic values of several representative immune subtypes ($CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ and $CD1a^+$ cells). We found that tumor-infiltrating $CD1a^+$ dendritic cells (DCs) and $CD8^+/FoxP3^+$ ratios were associated with favorable clinical outcomes and could potentially serve as predictors for clinical outcomes in TSCC patients.

Material and Methods

Patient Population

This study was approved by the Research Ethics Committee of Nanjing Stomatology Hospital, Nanjing University, P. R. China. Written informed consent was obtained from all of the patients. All specimens were handled and made anonymously according to the ethical and legal standards. The study comprises 153 patients from 2000 to 2011 diagnosed with TSCC except base of tongue cancer. All the slides of TSCC were evaluated according to WHO classifications by two pathologists, with differences resolved by careful discussions. The clinical stage was classified according to the 2002 UICC-TNM staging system. The clinical pathological features were shown in Table 1. None of the patients received chemotherapy or radiotherapy prior to surgery. 41 patients were lost to follow-up and 112 patients were followed up until 1 January 2014.

Evaluation of Tumor Inflammatory Infiltration

The rates of inflammation were evaluated based on hematoxylin and eosin staining slides according to the recommendation by the International TILs Working Group [17, 18]. Specifically, tumor area excluding immune infiltrates outside the tumor was selected to assess. Then, the percentage of the tumor stroma that was occupied by immune cells was estimated by the pathologists. <50% was defined as light to moderate inflammatory infiltrates and > 50% was defined as intense inflammatory infiltrates [18].

Determination of $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ Tumor Infiltrating Lymphocytes (TILs) and $CD1a^+$ DCs by Immunohistochemistry

A standard streptavidin-biotin peroxidase method was employed on 3 μm formalin fixed, paraffin embedded sections. The sections were stained with the mouse monoclonal antibodies anti-CD3 (dilution, 1:300; NCL-L-CD3–565; Novocastra Laboratories), anti-CD4 (dilution, 1:300; NCL-L-CD4–368; Novocastra Laboratories), anti-CD8 (dilution, 1:300; NCL-L-CD8–295; Novocastra Laboratories), anti-FoxP3 (dilution 1:500, SC-56680; Santa Cruz), anti-CD1a (dilution, 1:500; ab201337; Abcam). All sections were subsequently incubated with biotinylated secondary anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA, U.S.A.) followed by incubation with the avidin-biotin-complex-PO using the VECTASTAIN Elite ABC kit (Vector Laboratories) and developed in DAB. Positive staining of CD3, CD4, CD8 and CD1a were defined by the yellow to brown of cell membrane stain and the positive staining of FoxP3 was defined by the yellow to brown of nuclear stain. The tumor infiltrating cell number of $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ and $CD1a^+$ were evaluated by two pathologists, blinded for clinical outcomes. Each counted the number of cells located in tumor nest and tumor stroma in 5 randomly selected high-power fields (400×). The median was defined as cutoff value, which was 60, 22, 26, 10, 15, 0.73 and 2.52 for CD3, CD4, CD8, FoxP3, CD1a, CD4/CD8 and CD8/FoxP3, respectively. Low tumor infiltration was defined as score below the cutoff value, while high tumor infiltration was defined as score equal to or above the cutoff value.

Statistical Analysis

Statistical analyses were performed using the statistical software SPSS 17 (SPSS, USA) and Prism statistical software package (version 5.0, Graphpad Software Inc.). The descriptive statistical tests, including the mean and standard deviation were calculated according to the standard methods. For the correlations between tumor-infiltrating number of $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ T cells, $CD1a^+$ DCs and clinical pathologic features in TSCC were analyzed with Mann-Whitney U test. Overall survival (OS) and disease-free survival (DFS) comparing low and high tumor infiltration patients were estimated using Kaplan–Meier curves. Survival time was defined as the interval between the date of surgery and the last date when the patient was known to be disease-free or alive (censored). Cox

Table 1 Clinical characteristics of 153 TSCC patients

Factors		Number (range)	Percentage(%)
Age	Mean(range)	56.4 (20–84)	
Gender	Male	74	48.37
	Female	79	51.63
Smoking	Yes	56	36.60
	No	97	63.40
Differentiation	Well	60	39.22
	Medium to poor	93	60.78
Stage	I-II	71	46.41
	III-IV	82	53.59
Lymph node metastasis	No	93	60.78
	Yes	60	39.22
Tumor inflammatory infiltrate	Light to moderate	83	54.25
	Intense	70	45.75

proportional hazards regression was used for univariate and multivariate analysis of OS and DFS according to CD1a$^+$ cells infiltration and CD8$^+$/FoxP3$^+$ ratios. Differences were considered statistically significant with $P < 0.05$.

Results

Patient Outcomes

153 TSCC patients including 74 male and 79 female from Nanjing stomatology Hospital between 2000 and 2011 were involved in this study. The tumors were all located in the tongue including the front and the lateral except the base. Clinical, pathological and immunochemical characteristics at the time of diagnosis were shown in Table 1. The average age of the patients was 56.4 years.

The panel consisted of approximately equal number of Stage I/II ($n = 71$, 46.41%) and Stage III/IV ($n = 82$, 53.59%) tumors. 60 (39.22%) patients were with lymph node metastasis. Close to equal number of patients with light to moderate ($n = 83$, 54.25%) and intense inflammatory infiltration ($n = 70$, 45.75%) were studied in this research.

Correlations between the Number of CD3$^+$, CD4$^+$, CD8$^+$, FoxP3$^+$ and CD1a$^+$ Tumor Infiltrating Immune Cells and Clinicopathological Parameters in TSCC

Results showed that higher tumor inflammatory infiltration was associated with prolonged OS and DFS in TSCC (Fig. 1a, b). Since tumor inflammatory infiltration contains the immune cells including T cells and DCs, we analyzed the presence of T cells and DC cells in TSCC.

Under a high-power view, positive staining of CD3, CD4, CD8 and CD1a appeared as membranous while FoxP3 as nuclear staining (Fig. 2). These positive cells were predominantly observed in tumor stroma and few were found in tumor nest.

Results showed that the number of CD3$^+$, CD4$^+$ TILs and CD1a$^+$ DCs were higher in well differentiated TSCC patients compared with medium to poorly differentiated TSCC samples ($P < 0.001$, $P < 0.001$, $P < 0.001$) (Table 2), while the number of FoxP3$^+$ TILs was less in well differentiated TSCC patients compared with medium to poorly differentiated TSCC samples ($P < 0.001$) (Table 2). Furthermore, increased CD1a$^+$ DCs and CD8$^+$/FoxP3$^+$ ratios were associated with early clinical stages ($P < 0.01$, $P < 0.05$) (Table 2). However, high number of FoxP3$^+$ cells were associated with late clinical stages ($P < 0.01$) (Table 2). These results all suggested that FoxP3$^+$ cells, CD1a$^+$ cells and CD8$^+$/FoxP3$^+$ ratios were closely correlated with TSCC progression.

The Correlation between CD1a$^+$ DCs and TILs

As DCs are key regulators of T cell mediated immune responses, the correlations between TILs and DCs were then analyzed. We found the infiltration of CD3$^+$ and CD4$^+$ cells were positively related to CD1a$^+$ DCs ($P < 0.0001$; $P < 0.0001$; Fig. 3a, b). However, levels of CD8$^+$ and FoxP3$^+$ cells were negatively related to CD1a$^+$ DCs ($P < 0.001$; $P < 0.0001$; Fig. 3c, d).

DCs were found to activate and induce CD8$^+$ T cells, CD4$^+$ T cell as well as FoxP3$^+$ Treg cells [19]. Therefore, we further used CD1a$^+$/FoxP3$^+$ ratios as the indicators of the induction of Tregs by DC cells. Moreover, the correlations of CD3$^+$, CD4$^+$ and CD8$^+$ cells to ratio of CD1a$^+$/FoxP3$^+$ were investigated.

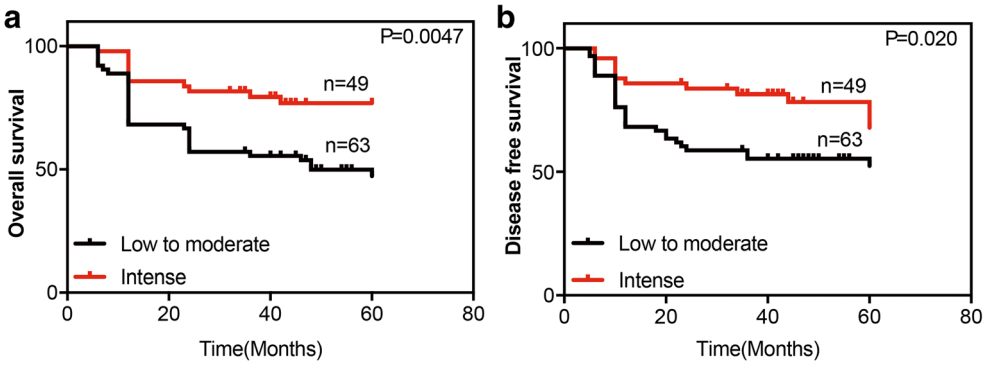

Fig. 1 Kaplan-Meir survival analyses of TSCC patients based on tumor inflammatory infiltration. (**a**) Kaplan-Meier analysis for OS of TSCC patients based on tumor inflammatory infiltration; (**b**) Kaplan-Meier analysis for RFS of TSCC patients based on tumor inflammatory infiltration

As shown in Fig. 3e, f and g, $CD3^+$ and $CD4^+$ cells were positively related to the ratios of $CD1a^+/FoxP3^+$, but $CD8^+$ cells were negatively related to the ratios of $CD1a^+/FoxP3^+$ ($P < 0.0001$; $P < 0.0001$; $P < 0.01$).

Tumor Infiltrating $CD8^+/FoxP3^+$ Ratios and $CD1a^+$ DCs Predicted Favorable Clinical Outcomes in TSCC Patients

As shown in Fig. 1, the patients with intense inflammatory infiltration had longer OS and DFS than the patients with low or moderate inflammatory infiltration ($P = 0.0047$; $P = 0.02$). Therefore, the prognostic values of TILs and DCs were analyzed in TSCC patients, respectively. When dichotomizing the patients using the median number of $CD1a^+$ DCs, the group with high $CD1a^+$ DCs had improved DFS ($P = 0.037$) (Fig. 4). In addition, the groups with higher $CD8^+/FoxP3^+$ ratios had better OS and DFS ($P = 0.047$, $P = 0.033$) (Fig. 4c, d). However, there were no significant differences between levels of $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ cells and $CD4^+/CD8^+$ ratios in relation to OS and DFS (Fig. S1).

The Ratios of $CD8^+/FoxP3^+$ T Cells and $CD1a^+$ DCs Levels Were Not Independent Prognostic Factors for TSCC Patients

Although higher levels of $CD1a^+$ DCs and $CD8^+/FoxP3^+$ ratios were associated with favorable clinical outcomes, the prognostic values should be further assessed when adjusted for age, gender, smoke, TNM, inflammation and tumor differentiation (Table 3). We evaluated the prognostic values of $CD1a^+$ DCs levels and $CD8^+/FoxP3^+$ ratios using multivariate Cox regression analysis. The multivariate regression analyses showed that the infiltration of immune cells including $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ TILs, $CD1a^+$ DCs as well as the ratios of $CD4^+/CD8^+$ and $CD8^+/FoxP3^+$ T cells were not independent prognostic factors. However, inflammation rates were independent prognostic factors for OS and DFS.

Discussion

TSCC, one of the most common OSCC, is characterized with a relatively poor prognosis [20]. Identifying reliable

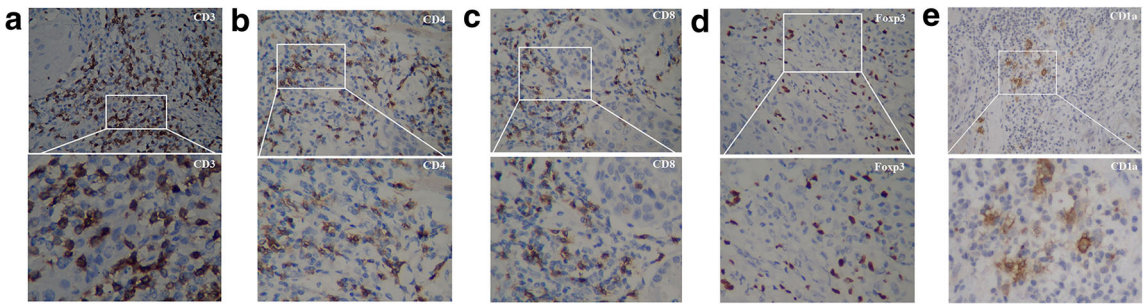

Fig. 2 Immunohistochemical staining of $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ and $CD1a^+$ tumor infiltrating immune cells. (**a**) Representative images of $CD3^+$ immunohistochemical stain in magnification 10× (upper) and 20× (below). (**b**) Representative images of $CD4^+$ immunohistochemical stain in magnification 10× (upper) and 20× (below). (**c**) Representative images of $CD8^+$ immunohistochemical stain in magnification 10× (upper) and 20× (below). (**d**) Representative images of $FoxP3^+$ immunohistochemical stain in magnification 10× (upper) and 20× (below). (**e**) Representative images of CD1a+immunohistochemical stain in magnification 10× (upper) and 20× (below)

Table 2 Correlations between tumor-infiltrating CD3$^+$, CD4$^+$, CD8$^+$ and FoxP3$^+$ Treg T cell subsets, CD1a$^+$ DCs and the clinicopathologic features in TSCC

Patients and tumor characteristics	*n*	CD3$^+$ (mean ± SD)	*P*	CD4$^+$ (mean ± SD)	*P*	CD8$^+$ (mean ± SD)	*P*	CD4/CD8 (mean ± SD)	*P*	FoxP3$^+$Treg (mean ± SD)	*P*	CD1a$^+$ DC (mean ± SD)	*P*	CD8/FoxP3 (mean ± SD)	*P*
Sex															
Male	74	57.01 ± 8.93	**<0.05**	20.77 ± 6.66	<0.05	28.07 ± 6.72	>0.05	0.80 ± 0.39	<0.05	13.30 ± 7.16	>0.05	16.84 ± 11.10	>0.05	2.89 ± 2.02	>0.05
Female	79	60.24 ± 8.99		23.77 ± 8.08		27.03 ± 7.32		0.97 ± 0.48		12.38 ± 7.91		18.11 ± 8.33		2.99 ± 1.52	
Age															
<60	90	59.09 ± 9.32	>0.05	22.41 ± 7.80	>0.05	27.81 ± 7.47	>0.05	0.89 ± 0.48	>0.05	13.13 ± 7.75	>0.05	17.89 ± 10.42	>0.05	2.95 ± 1.93	>0.05
≥60	63	58.10 ± 8.76		22.19 ± 7.26		27.13 ± 6.39		0.87 ± 0.40		12.38 ± 7.27		16.94 ± 8.78		2.91 ± 1.54	
Smoking															
No	97	58.79 ± 9.40	>0.05	23.01 ± 8.08	>0.05	26.57 ± 7.39	**<0.05**	0.96 ± 0.50	**<0.05**	12.71 ± 8.12	>0.05	18.80 ± 8.30	>0.05	2.90 ± 1.65	>0.05
Yes	56	58.48 ± 8.57		21.13 ± 6.44		29.20 ± 6.07		0.76 ± 0.31		13.02 ± 6.75		16.64 ± 11.90		3.01 ± 1.98	
Stage															
I-II	71	58.72 ± 8.67	>0.05	23.13 ± 7.54	>0.05	27.01 ± 761	>0.05	0.96 ± 0.50	>0.05	11.23 ± 7.11	**<0.01**	20.08 ± 10.71	**<0.01**	3.18 ± 1.69	**<0.05**
III-IV	82	58.65 ± 9.47		21.62 ± 7.55		27.98 ± 6.50		0.83 ± 0.40		14.21 ± 7.68		15.26 ± 8.28		2.73 ± 1.83	
Differentiation															
Well	60	66.20 ± 7.14	**<0.001**	29.10 ± 6.21	**<0.001**	27.22 ± 9.23	>0.05	1.22 ± 0.50	**<0.001**	6.28 ± 2.14	**<0.001**	26.20 ± 9.18	**<0.001**	4.57 ± 1.60	**<0.001**
Medium to poor	93	53.83 ± 6.54		17.95 ± 4.51		27.73 ± 5.19		0.67 ± 0.24		17.04 ± 6.72		11.88 ± 4.68		1.89 ± 0.84	
Lymph node metastasis															
No	93	58.72 ± 9.60	>0.05	23.07 ± 8.44	>0.05	26.72 ± 6.57	>0.05	0.93 ± 0.47	>0.05	12.03 ± 7.11	>0.05	17.82 ± 9.44	>0.05	3.04 ± 1.81	>0.05
Yes	60	58.63 ± 8.39		21.31 ± 6.08		28.63 ± 7.53		0.82 ± 0.41		13.89 ± 8.03		17.06 ± 10.22		2.80 ± 1.73	
Tumor inflammatory infiltrate															
Light to moderate	83	58.67 ± 9.83	>0.05	22.04 ± 7.66	>0.05	28.18 ± 6.89	>0.05	0.84 ± 0.40	>0.05	12.34 ± 6.88	>0.05	17.25 ± 9.94	>0.05	3.03 ± 1.72	>0.05
Intense	70	58.69 ± 8.17		22.66 ± 7.48		26.76 ± 7.17		0.94 ± 0.50		13.40 ± 8.28		17.79 ± 9.59		2.83 ± 1.84	

Bold values indicate the significant difference at the level of 0.05

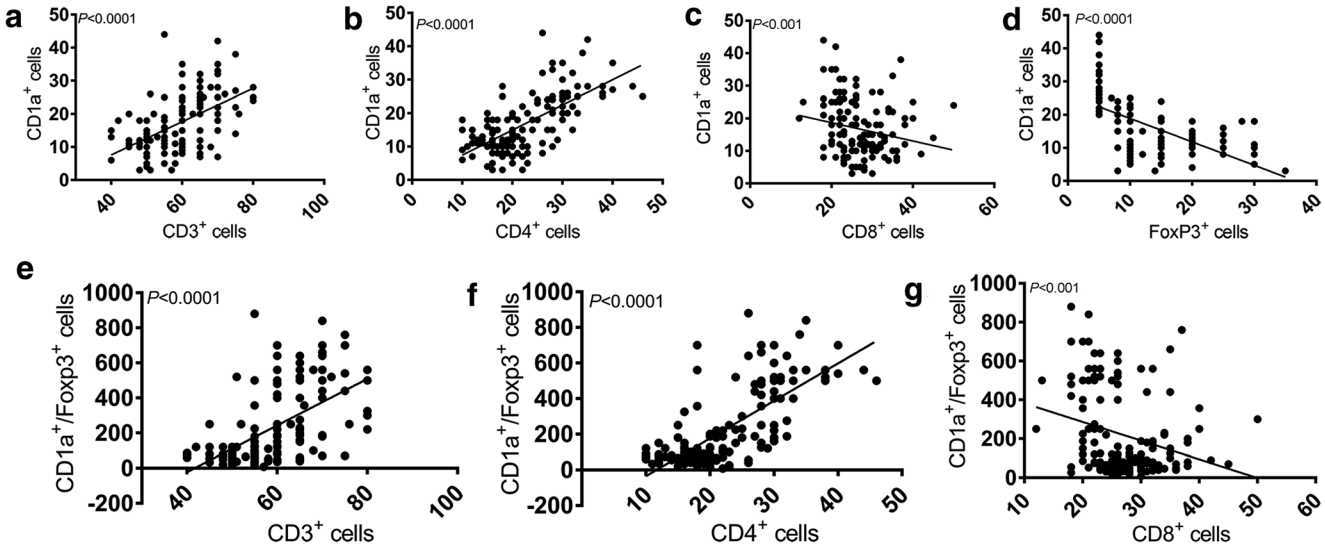

Fig. 3 Spearman correlation between tumor infiltrating immune cells. (**a**) Correlation between $CD3^+$ and $CD1a^+$ cells; (**b**) Correlation between $CD4^+$ and $CD1a^+$ cells; (**c**) Correlation between $CD8^+$ and $CD1a^+$ cells; (**d**) Correlation between $FoxP3^+$ and $CD1a^+$ cells; (**e**) Correlation between $CD3^+$ cells and$CD1a^+/FoxP3^+$ ratio; (**f**) Correlation between $CD4^+$ cells and $CD1a^+/FoxP3^+$ ratio; (**g**) Correlation between $CD8^+$ cells and $CD1a^+/FoxP3^+$ ratio

prognostic markers will guide TSCC treatments. In this study, we evaluated the density and prognostic values of tumor-infiltrating immune cells, including $CD3^+$, $CD4^+$, $CD8^+$, $FoxP3^+$ and $CD1a^+$ cells and found that the patients with more $CD1a^+$ cells infiltration and higher $CD8^+/FoxP3^+$ ratios displayed favorable clinical outcomes.

Head and neck squamous cell carcinoma (HNSCC) comprises tumors of diverse origins. In spite of their differences in clinical behaviors, HNSCC patients with different anatomical subsites were rarely studied separately. However, TSCC should be studied separately as it has unique epidemiological characteristics different from those other oral cavity cancers [21, 22]. Therefore, in this study a homogenous cohort of TSCC patients (excluding base of tongue cancer) was selected to study various immune parameters. Although the scale of this study was not large enough, it was still convincing because it had a consecutive 5-year period follow up and was the largest study of tumor infiltrating immune cells exclusively on tongue cancer patients in China.

Lymphocyte infiltration is the result of tumor host interaction. In this study, tumor inflammatory infiltrate levels were associated with promising clinical outcomes as patients with intense infiltrates survived longer and recurrence was prolonged. In addition, inflammation was an independent prognostic factor for OS and DFS, which was consistent with previous findings [23, 24]. However, there were some contradictory findings [25, 26]. As tumor-infiltrating immune cells are a heterogeneous population of cells with both pro-tumoral and anti-tumoral effects, it was unreliable to use inflammatory infiltrates as prognostic biomarkers for TSCC patients. Therefore, we further analyzed the correlation between several immune subsets and clinical outcomes, respectively.

DCs are responsible for linking innate to adaptive immunity. CD1a is widely used as immature DC marker, such as Langerhans cells. Previous studies showed that levels of $CD1a^+$ were lower in lip squamous cell carcinoma than normal epithelium, which suggested that the inadequate antigen presentation was important for cancer development [27]. In addition, high counts of DCs infiltration were found to be associated with longer DFS in laryngeal squamous cell carcinoma and OSCC [28, 29]. In our study, we found that $CD1a^+$ cell levels were not only correlated with well differentiation and early clinical stages, but also with improved DFS in TSCC, suggesting that CD1a was a potential prognostic marker in TSCC.

T cells are a heterogeneous group of cells with several distinct populations but all express CD3. $CD4^+$ T cells are one subset of T cells which not only contribute to antitumor responses but also to tumor-promoting activities. Therefore, it is ambiguous relating to the association between $CD3^+$ and $CD4^+$ T cells and clinical outcomes as contradicting findings were reported in OSCC [14, 30–32]. In our 153 TSCC patients, we found that $CD3^+$ and $CD4^+$ T cell levels were correlated with tumor differentiation and no prognostic values concerning OS or DFS were found. Considering the mixed subpopulation of $CD3^+$ and $CD4^+$ cells with both pro-tumor and anti-tumor effects and the ambiguous conclusions from previous studies, the prognostic role of CD3 and CD4 remains questionable and a more specific classification of these cells is required.

Table 3 Summary of multivariate Cox regression analysis of overall survival and disease free survival

Variables	OS			DFS		
	HR	Multivariate 95% CI	Sig.	HR	Multivariate 95% CI	Sig.
Gender						
Female vs. Male	0.448	0.17–1.19	0.108	0.746	0.26–2.14	0.586
Age						
<55 vs. ≥55	1.787	0.91–3.53	0.093	2.509	1.25–5.03	**0.009**
Smoke						
Yes vs. No	0.543	0.19–1.56	0.257	0.823	0.26–2.59	0.74
TNM						
I-IIvs.III-IV	0.502	0.14–1.85	0.301	0.704	0.22–2.28	0.559
Differentiation						
Low vs. Moderate-High	0.282	0.07–1.11	0.07	0.274	0.073–1.03	0.055
Inflammation						
Light to moderate vs. intense	0.332	0.15–0.76	**0.009**	0.397	0.17–0.93	**0.033**
CD3						
Low vs. High	0.828	0.38–1.82	0.638	0.931	0.41–2.12	0.865
CD4						
Low vs. High	1.062	0.44–2.60	0.895	1.564	0.61–3.99	0.35
CD8						
Low vs. High	0.948	0.40–2.26	0.903	1.187	0.49–2.88	0.705
Treg						
Low vs. High	1.519	0.53–4.35	0.436	1.701	0.55–5.27	0.357
CD1a						
Low vs. High	0.768	0.32–1.84	0.553	0.586	0.24–1.43	0.24
CD4/CD8						
Low vs. High	1.228	0.47–3.18	0.672	1.211	0.46–3.22	0.701
CD8/Treg						
Low vs. High	0.459	0.13–1.69	0.241	0.424	0.11–1.60	0.205

Abbreviations: *CI* confidence interval, *HR* hazard ratio, *OS* overall survival, *DFS* disease-free survival. Figures in boldface indicate statistical significance

Tregs are one class of CD4$^+$ T cells, which expressed FoxP3 and CD25. The transcription factor FoxP3 is the most widely used specific marker for Tregs so far. In context of tumors, Tregs cells are associated with facilitating the suppression of the antitumor responses. Therefore, FoxP3$^+$ cells proved an unfavorable factor in oral cavity cancers

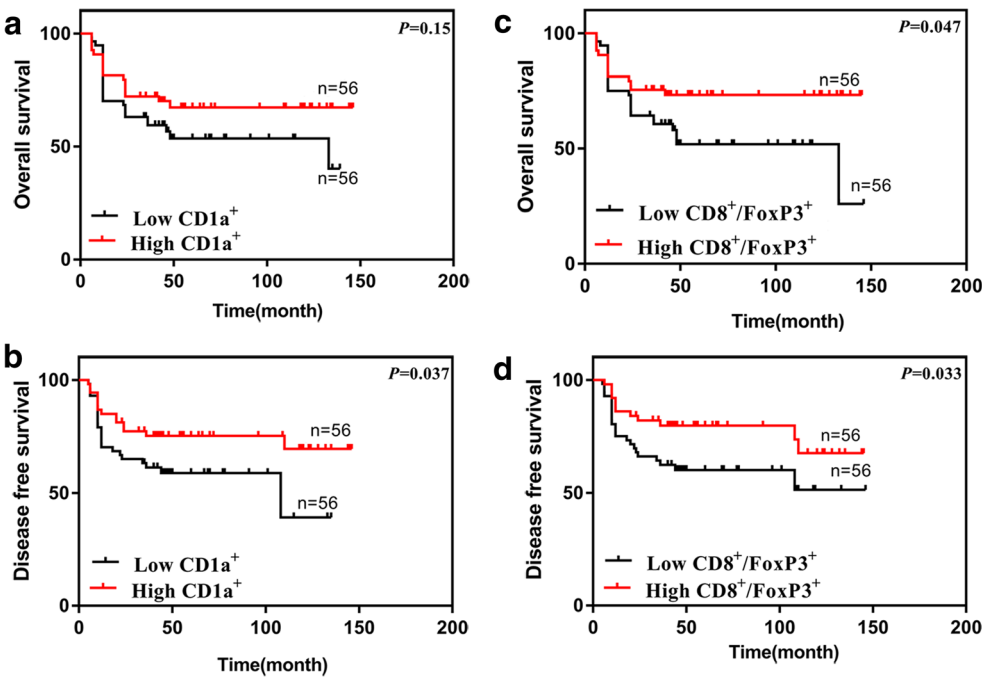

Fig. 4 Kaplan–Meier plots of over survival, relapse free survival and disease free survival of TSCC patients according to the infiltration of TILs. (**a**) OS and DFS of TSCC patients with CD1a$^+$ infiltration above and below median. (**b**) OS and RFS of TSCC patients with CD8$^+$/FoxP3$^+$ ratio above and below median

[32]. However, there are many instances where FoxP3$^+$ levels linked to favorable clinical outcomes [11, 13, 30]. We found that FoxP3$^+$ cells were related to unfavorable clinical factors, but not associated with OS and DFS. The conflicting results from different studies suggested that FoxP3 was over-simplified in the current measurement models as FoxP3$^+$ cells act differently in different anatomical subsites and tumor stages. It is suggested that FoxP3$^+$ cells infiltration reflected the total amount of T cells in the epithelium which may explain its favorable clinical outcomes and investigation of CD8$^+$/FoxP3$^+$ ratios may help determine whether pro-tumor effects outweigh anti-tumor effects [7]. Therefore, we then analyzed the CD8$^+$/FoxP3$^+$ ratios. Generally, higher number of intra-tumoral CD8$^+$ cytotoxic T-cells is associated with better clinical outcomes [5, 7]. However, in our study no association between CD8$^+$ levels and patients' survival was found. Higher ratios of CD8$^+$/FoxP3$^+$ were associated with improved OS and DFS in our study, which was consistent with Chen's and Näsman's findings [33, 34]. The ratios of CD8$^+$/FoxP3$^+$ proved to be an independent prognostic factor in a number of tumors, including colorectal cancer and breast cancer [35]. Therefore, the ratio of CD8$^+$/FoxP3$^+$ was a promising prognostic marker in TSCC.

Although we used a more homogenous cohort of tumors from tongue, we failed to separate patients relating to tumor stage and HPV infection status, which were reported to be associated with immune cells infiltration. The use of more homogeneous patient cohorts would strengthen the conclusions on prognostic biomarkers and also provide more insights into the differences between patient subgroups.

In conclusion, our study revealed that the immune cells infiltration predicted favorable clinical outcomes. Particularly, levels of CD1a$^+$ cells and CD8$^+$/FoxP3$^+$ ratios were associated with favorable clinical outcomes, although they were not independent prognostic factors. The results suggested that based on tumor immune cells infiltration levels, it is possible to provide new clues to therapeutic strategies.

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Compliance with Ethical Standards

Conflict of Interest None declared.

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