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Original Article

Evaluation of CD4+ tumor-infiltrating lymphocyte association with some clinicopathological indices of oral squamous cell carcinoma

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ABSTRACT

Background: The delayed diagnosis of oral squamous cell carcinoma (OSCC) affects therapeutic and prognostic strategies, and provides regional recurrence or distant metastasis. The tumor-infiltrating lymphocytes (TILs) are known as a critical diagnostic biomarker in antitumor immune response. We evaluated the association between CD4+ T-lymphocyte marker, some clinicopathological indices, and the impact of TILs on the stage and grade of OSCC.

Materials and Methods: In this cross-sectional study, 37 OSCC specimens including 16 early and 21 advanced stages (categorized base-on recent clinical oncology references) and their related healthy surgical margin (as internal control group) were collected. Obtained histochemical data were analyzed by SPSS V.23 software. The expression of CD4+ marker in tumor microenvironment (TME) was compared by nonparametric Mann–Whitney and Kruskal–Wallis as well as Fisher's exact tests. $P < 0.05$ was remarked statistically significant.

Results: The low-grade patients represented more CD4+ TIL that was statistically significant ($P = 0.011$). However, there was no statistically significant difference in CD4+ TIL between various stages ($P = 0.404$), tumor size, and lymph node involvement ($P > 0.05$). Moreover, there was no significant relation between TIL infiltration, age, and tumor localization ($P > 0.05$), however CD4+ expression in women was more than men ($P = 0.008$). The CD4+ T-lymphocyte infiltration in TME was more significant than healthy surgical margin ($P < 0.001$). There was a statistically significant difference between healthy surgical margin and different grades and stages of OSCCs that lower grades demonstrated more CD4+ TIL infiltration ($P < 0.001$).

Conclusion: The CD4+ T-lymphocytes may play important role in differentiation and maturity of epithelial cell, tumorigenesis, and progression of OSCC.

Key Words: CD4+ T-lymphocyte, diagnosis, oral cancer, prognosis, tumor-infiltrating lymphocyte

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for 90% of all head-and-neck squamous cell

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carcinomas (HNSCCs) as the first common location and known as the seventh most frequent tumor with poor survival rates.[1,2] HNSCC comprised vast tumors that are derived from the mucosal epithelium in the oral cavity, pharynx, larynx, nose, nasal cavity, and paranasal sinuses. Tobacco consumption, alcohol abuse, or both and HPV infection are the main high-risk factors for tumorigenesis.[3-5] The anatomical location of the HNSCC provide late diagnosis, this problem affects therapeutic and prognostic strategies, and appears regional recurrence or distant metastasis. The traditional first-line therapy including extended surgery with tumor margins and lymph node resection, radiotherapy, and chemotherapy restricted improvement in long-term survival but recent strategies based on target therapy associated with immune modulation and tumor microenvironment (TME) that showed improvement in outcome for HNSCC patients in some research studies.[6,7]

Previous studies demonstrated immune cells involved in tumorigenic process and ongoing trials apply immunotherapeutic approaches for prevention of HNSCC development and progression.[8] Assessment of cancer biomarkers helps us for better estimation of prognosis and prediction of response to therapy. It was shown evaluation of tumor-infiltrating lymphocytes (TILs) can be applied as promising prognostic biomarkers in solid tumors such as carcinomas of the lung, gastrointestinal tract, genitourinary system, and brain; melanoma; and HNSCCs.[9] The TILs including B-, T-, and NK cells. These cells in region of tumor known as a critical biomarker in antitumor immune response and affect cancer invasion and metastasis.[10] TILs can be targeted for new OSCC immunotherapy purposes.[2,11,12]

Evaluation of TILs and their T-cell subsets demonstrated an increase in CD4, CD8, and FOXP3 density and upregulation of the molecular signature in OSCC patients.[13] It seems we can consider different TIL types in estimation of prognosis and evaluation of clinicopathological indices of OSCC, because a decrease in TIL levels is in correlation with overall survival (OS) and disease-specific survival.[14] In this study, we evaluate the association between CD4 marker in T-cells and clinicopathological indices in patients with OSCC for better prognosis, and the impact of TILs on tumor stage, grade, and progression.

MATERIALS AND METHODS

Study population

In this cross-sectional study, 37 OSCC paraffin blocks including 15 women and 22 men with their related healthy surgical margins (internal control group) were collected from the Department of Oral and Maxillofacial Pathology, School of Dentistry, Mashhad University of Medical Sciences, Mashhad, Iran. The samples were obtained from the year 2017 to 2020. The use of OSCC samples was approved by the Mashhad University Ethics Committee (IR.MUMS.DENTISTERY.REC.1399.066), and written consent was obtained from all participants. The inclusion criteria were OSCC paraffin blocks with efficient quality and primary tumors but not recurrence cases. All of OSCC patients' demographic information and medical history were completely registered. The patients who previously underwent surgery, radiotherapy, or chemotherapy were excluded from this study. The samples demonstrated low quality, unsuitable fixation, necrotic or bloody district were excluded from this study, too.

Tissue samples

For all of patients fully examining was performed and their demographic information was registered. All collected tissue samples were fixed in 10% neutral-buffered formalin solution at room temperature for 72 h, and then samples were embedded in paraffin block. Next, the paraffin blocks were cut off into section with 1-μm thickness. Two sections are needed for H and E staining to approve the diagnosis of tumor samples based on their stage and grade.

Immunohistochemically staining: Heat-induced epitope retrieval technique and peroxidase antiperoxidase technique

The 4-μm tissue sections were placed on glass slides covered by poly-L-lysin. Next, deparaffinized with xylene and rewashed by alcohol to water for 5 min. The antigens were retrieved with Tris-EDTA buffer (pH = 8) at 98°C for 20 min. They cooled up at RT for 20 min and washed with TBS buffer and lied on Hydrogen peroxide solution 3% to reduce background reaction for 10 min. Next, the slides were rinsed up with TBS buffer again. The primary CD4 antibody (MAD-000600QD, Master Diagnose, Spain) was ready to use, they were incubated with CD4 antibody for 40 min and rewashed with TBS, and the procedure was performed based on protocol with optimal laboratory condition. Then, postprimary

block solution (Leica Co, United Kingdom) was used for 20 min. The slides were rewashed with TBS again. Next, the slides were incubated with Novolink Polymer (DAB included, Germany) for 20 min. After rewashing by TBS buffer, the slides were incubated with diaminobenzidine peroxidase substrate (ref. MAD-021540Q-125). Then, these slides washed by tap water, and placed on hematoxylin for 3 min. Then these slides washed by tap water, and rewashed through graded alcohol and the slides evaluated with light microscopy.

The tissue sections were evaluated by an expert pathologist for TILs by CD4 marker. Pathologist in five fields of view were observed for each section under a lens (100 X). The TIL assessment was performed based on the tumor infiltration staining was scored accordingly: absence (lower than 5 positive cells: score 0), low (5–25 positive cells: score 1), moderate (25–75 positive cells: Score 2), and strong (more than 75 positive cells: score 3).[15-17] The base of T CD4+ lymphocyte infiltration was represented by counting of these cells at invasive front of cancer cells in stroma. Some stained slides were captured by LABOMED optical microscope and TrueChrome Metrics (EX Fucos 0.5X, USA)[18,19] [Figure 1]. The CD4-positive control samples were tonsil tissues and negative control samples were hepatocellular carcinoma for immunohistochemically staining (IHC).

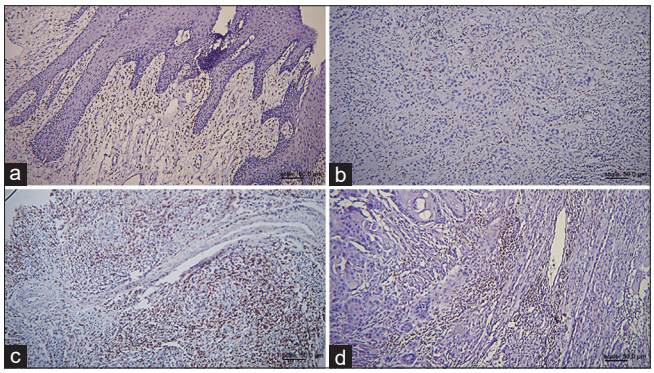

Figure 1: Immunohistochemically staining of CD4+ T-lymphocyte expression in tumor tissues and healthy surgical margin. (a) Immunohistochemically staining of healthy surgical margin in SCC (Grade I) demonstrated CD4+ expression with Score II (X200). (b) Immunohistochemically staining of SCC (Grade III) demonstrated CD4+ expression with Score I (×100). (c) Immunohistochemically staining of SCC (Grade II) demonstrated CD4+ expression with score III (×100). (d) Immunohistochemically staining of SCC (Grade I) demonstrated CD4+ expression with score III (×100). SCC: Squamous cell carcinoma.

Statistical methods

The demographic information of HNSCC patients and result of IHC staining were analyzed by SPSS software, version 22 (SPSS Inc., Chicago, IL, USA). The expression of CD4+ marker and the staining strength in TME were compared by nonparametric Mann–Whitney and Kruskal–Wallis as well as Fisher's exact tests. A $P < 0.05$ was remarked statistically significant.

RESULTS

In this study, 37 patients including 15 women (40.5%) and 22 men (59.5%) participated. The mean age was between 60.00 ± 14.4 years with 34–84 range. Eighteen cases (48.6%) were histopathologically Grade I, 15 cases (40.5%) were Grade II, and 4 cases (10.8%) were Grade III. The grading classification was performed according to some indices of the College of American Pathologists (CAP) protocols.[20] Twenty-one patients (56.8%) demonstrated lymph node involvement and 12 cases (32.4%) showed distant metastasis. Sixteen cases (43.2%) were in early stage and 21 cases (56.8%) were in advanced stage. The stages were classified base-on valid previous articles.[19,21] The size of tumor in 22 patients (59.5%) was 2 cm ≥ and in 78 cases (40.5%) was more than 2 cm. Seventeen samples (45.9%) were from tongue, 7 cases (18.9%) from lips, 5 cases (13.6%) from posterior region of oral cavity, and 8 cases (21.6%) from gum. Nine patients (24.3%) showed cellularity with score 1, 15 patients (40.5%) demonstrated score 2, and 13 cases (35.1%) exhibited score 3. In healthy surgical margin tissue, 24 cases (64.9%) showed the absence of CD4, 9 cases (24.3%) demonstrated score 1, 1 case (2.7%) exhibited score 2, and 3 cases (8.1%) showed score 3 [Table 1].

All tumoral tissues demonstrated different scores, but 24 samples (64.9%) of 37 healthy surgical margin tissues showed negative CD4 expression. The expression of CD4 marker in tumoral tissues was statistically significant in comparison to the healthy surgical margin ($P < 0.001$) [Table 2].

The CD4+ T-lymphocytes showed a higher score in lower grades and a lower score in higher grades. The grades demonstrated a statistically significant difference in CD4+ T-cell expression ($P = 0.011$) [Table 3].

In early stage, the half of CD4+ T-lymphocytes were score 2, 12.5% were score 1, and 37.5% were

Table 1: Profile information of the study participants

Variants	Count (%)
Sex	
Men	22 (59.5)
Women	15 (40.5)
Grade	
I	18 (48.6)
II	15 (40.5)
III	4 (10.8)
Lymph node involvement	
No	16 (43.2)
Yes	21 (56.8)
Distant metastasis	
No	25 (67.6)
Yes	12 (32.4)
Stage	
Early	16 (43.2)
Advanced	21 (56.8)
Tumor size (cm)	
≤2	22 (59.5)
>2	15 (40.5)
Tumor localization	
Tongue	17 (45.9)
Lip	7 (18.9)
Posterior region of oral cavity	5 (13.6)
Gum	8 (21.6)
CD4+ T-cells in tumor tissue	
Low cellularity (Score I)	9 (24.3)
Moderate cellularity (Score II)	15 (40.5)
High cellularity (Score III)	13 (35.1)
CD4+ T-cells in margin	
Absent	24 (64.9)
Low cellularity (Score I)	9 (24.3)
Moderate cellularity (Score II)	1 (2.7)
High cellularity (Score III)	3 (8.1)

Table 2: Comparison of CD4+ expression in healthy surgical margin and tumoral tissues

Margin tissue	Tumoral tissue				Wilcoxon test
	Score I	Score II	Score III	Total	
Score 0	8 (33.3)	10 (41.7)	6 (25)	24 (100)	Z=4.89
Score I	0 (0)	5 (55.6)	4 (44.4)	9 (100)	P<0.001
Score II	0 (0)	0 (0)	1 (100)	1 (100)	
Score III	1 (33.3)	0 (0)	2 (66.7)	3 (100)	
Total	9 (24.3)	15 (40.5)	13 (35.1)	37 (100)	

score 3. While, in advanced stage, the expression of CD4+ T-lymphocytes was similar in all scores. No statistically significant difference observed between expression of CD4+ T-lymphocytes and early and advanced stages (P = 0.404) [Table 4]. The mean rank of healthy surgical margin's CD4+ T-lymphocytes was statistically significant

Table 3: The comparison of CD4+ T-lymphocyte expression in different grades

Grade	CD4+ T-lymphocyte count (%)			Total	Mean rank*	Kruskal–Wallis test
	Score I	Score II	Score III			
I	2 (11.1)	6 (33.3)	10 (55.6)	18 (100)	23.44[a]	χ^2=9.02
II	4 (26.7)	8 (53.3)	3 (20)	15 (100)	16.60[b]	P=0.011
III	3 (75)	1 (25)	0 (0)	4 (100)	8.00[c]	
Total	9 (24.3)	15 (40.5)	13 (35.1)	37 (100)		

*Similar lowercase letters indicate no significant differences between groups

Table 4: Comparison of CD4+ T-lymphocyte expression between early and advanced stages

Stage	CD4+ T-lymphocyte count (%)			Total	Mean rank	Mann–Whitney U-test
	Score I	Score II	Score III			
Early	2 (12.5)	8 (50.0)	6 (37.5)	16 (100.0)	20.75	Z=0.92
Advanced	7 (33.3)	7 (33.3)	7 (33.3)	21 (100.0)	17.67	P=0.404
Total	9 (24.3)	15 (40.5)	13 (35.1)	37 (100.0)		

in various stages (P < 0.001). The comparison of samples demonstrated that the mean rank in early and advanced stages was statistically significant in comparison to healthy surgical margin (P < 0.001), There was a statistically significant difference between healthy surgical margin and different grade and stage that lower grades demonstrated more TIL CD4+ infiltration (P < 0.001) [Figures 2 and 3].

The expression of T-lymphocytes with high cellularity was statistically significant in women in comparison to the men (P = 0.008). The expression of T-lymphocytes with high cellularity was higher in 58 ≥ years' age range than 58 < years, but it was not statistically significant difference (P = 0.258). The expression of T-lymphocytes with high cellularity without lymph node involvement was more than patients with lymph node involvement, but there were no statistically significant differences (P = 0.370). The expression of T-lymphocytes with high cellularity without distant metastasis was more than patients with distant metastatic cases, but there were no statistically significant differences (P = 0.620). The expression of T-lymphocytes with high cellularity in small tumor size was more than larger tumor size, but there was no statistically significant difference (P = 0.636). The expression of T-lymphocytes with high cellularity in tongue was more than the other location, but there was no statistically significant difference (P = 0.204). No statistically significant difference was between expression of CD4+ T-lymphocytes and high and low risks of IF (P = 0.819) and depth of invasion (P = 0.546). No

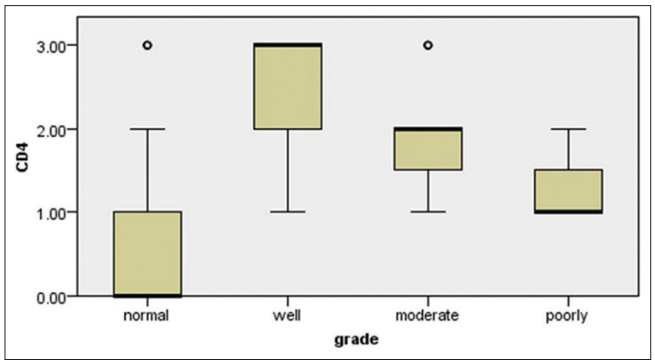

Figure 2: Distribution of CD4⁺ T-lymphocyte's healthy surgical margin in different grades of OSCC ($P < 0.05$). OSCC: Oral squamous cell carcinoma.

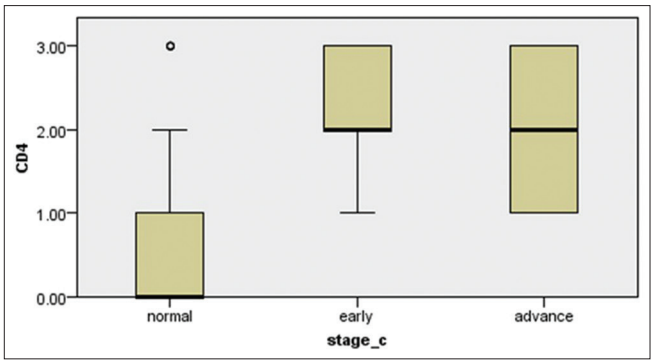

Figure 3: Distribution of CD4⁺ T-lymphocyte's healthy surgical margin in different stages of OSCC. OSCC: Oral squamous cell carcinoma.

statistically significant difference was between nerve invasion and noninvasion ($P = 0.719$), vascular invasion and noninvasion ($P = 0.729$).

DISCUSSION

Oral cancers are the sixth world's most common cancer.[22] Approximately 50% of patients encounter with treatment failure.[23,24] Today, research has proposed more conservative adjunctive therapies in therapeutic approaches of different cancers such as immunotherapy and target therapy, which have been successful in some tumors, such as melanoma.[25] However, there were no extensive studies about OSCC, and as our knowledge was obtained from valid available sources, there were restricted researches in immunotherapy and target therapy of OSCC patients. In this study, we estimated some indices of new grading protocol's system named CAP in OSCC patients that are determined base-on disease prognosis.[20] We evaluated some CAP indices including TME, lymph node involvement, and tumor size in the present study. We hope CAP be considered routine assessment method in research studies. The expression of CD4⁺ TILs was determined, and its association with grade and stage of OSCC patients was assessed. Our results approved the strong association of high CD4⁺ T-lymphocyte expression in low grades and representation of more CD4 marker expression in early-stage tumor. Although there were no statistically significant differences, the expression of CD4 marker was more in patients without lymph node involvement and distant metastasis and small size tumor groups in comparison to their opposition group.

However, there are controversial reports about prognostic value of CD4⁺ TILs as a biomarker for predicting HNSCC outcome, but it could not reduce the important role of immune cells in TME to inhibit tumor cell improvement or progression.[16,26] Previous studies have known TILs as a key modulator of cancer progression and mentioned a positive strong association between TIL subsets and OS in other cancers including cutaneous melanoma,[25] ovarian cancer,[27] breast cancer,[28] laryngeal cancer,[29] hypopharyngeal squamous cell carcinoma.[30] It was estimated 235 differentially expressed genes that involved in OSCC progression. Among 10 pivotal genes, there are three immune-related genes that their signature proposed potential predictor role of them in OSCC patient diagnosis, survival, prognosis, and therapy like T-cell CD4⁺ central memory.[31]

Although, there are few eligible reports and many publications bias in studies that present ambiguous role of CD4⁺ as prognostic biomarker. But still, it is questionable that whether CD4⁺ and the other CD8+, FoxP3+, and PD1⁺ TILs can provide the prognostic role. de Ruiter *et al.* in 2020 evaluated the CD4⁺ TILs in HNSCC HPV⁻ patients including 59.7% oropharyngeal, 32.3% hypopharyngeal, and 18.0% laryngeal cancers. Similar to our study, they reported no statistically significant difference between CD4⁺ TILs and sex, tumor location, size, age, and lymph node involvement.[32] In a recent study, Lequerica-Fernández *et al.* in 2021 demonstrated that CD8+, CD4+, and FoxP3⁺ TILs can target for therapeutic approaches, because there is a negative relationship between cancer stem cell markers such as NANOG and SOX2 expression and CD8⁺/CD4⁺ TILs.[33] Suárez-Sánchez *et al.* in 2021 confirmed a strong association between CD4+, CD8+, and FOXP3⁺ TILs and CD20⁺ B-lymphocyte expression that affect survival and prognosis in OSCC

patients, and also CD20+ B-lymphocytes showed a negative correlation with NANOG and SOX2.[34] It seems co-expression analysis of the other novel biomarker like aurora kinase A and ninein-interacting protein (AUNIP) besides that TILs have a potential prognostic biomarker for OSCC. Because AUNIP is in correlation with TILs and engages them to the TME in OSCC.[35] The association between valuable diagnostic and prognostic roles of AUNIP and CD4+/CD8+ T-lymphocytes was approved for the other tumors such as hepatocellular carcinoma and lung adenocarcinoma.[36]

The 5-year follow-up of HNSCC patients and evaluation of their immune-related gene signatures demonstrated significant infiltration of CD4+ T-lymphocytes. Association between developed immune-related signature and clinicopathological characteristics can help us to approximate more reliable OS.[37,38] In addition to microarray analysis, RNA seq and IHC staining manifested the correlation of TILs as prognostic biomarker factors in prediction of HNSCC. Dominant expression of CD4+ memory T-cells reflects the state of the immune microenvironment in HNSCC patients.[39,40] The transcriptomic analysis of more than 11,000 single T-cell of OSCC patients by RNA sequencing discovered that FOXP3 and CTLA4 were of CD4+ Treg markers.[41] The (IRGPI) is a potential prognostic indicator that determined by immune's tumor microenvironment, it can considered for evaluation of immune-related genes as prognostic index in HNSCC. Analysis of multi-omics data in high-IRGPI patients identified more OS with strong immune activity and minimum aggressive tumor features.[42] Result of IRGPI analysis highlighted the implementation of immunotherapy for HNSCC.

The results of Hu *et al.* in 2020 demonstrated that hypopharyngeal SCC patients represent a statistically significant difference of more CD4+ TILs in lower grade, however, there was no statistically significant difference in various stages. The CD4+ TILs were not demonstrated a statistically significant difference with tumor size and lymph node involvements. These results were similar to our study. However, no statistically significant difference was between men and women in CD4+ T-lymphocytes expression. According to our results, this difference can be related to the sample size of the study or racial difference.[43]

Schulze *et al.* in 2020 reported an association between high penetration of CD4+ TILs and advanced stage of non-small cell lung cancer and lymph node involvement stage I (19.8%), stage II (22.6%), stage III (26.2%), $P = 0.034$. Their results were different with us; in our study, the CD4+ TILs were more in nonlymph involvement patients but did not demonstrate a statistically significant difference. On the other hand, there was a statistically significant difference between CD4+ TILs and higher stages ($P = 0.047$). These differences might be due to the multiple malignancy's assessment in one location including adenocarcinoma, giant cell carcinoma, and squamous cell carcinoma, and also because of different biological behavior between non-small cell lung cancer and OSCC.[44]

Boxberg *et al.* in 2019 showed that high infiltration of T CD4+ FOXP3+ increased the ratio of CD4+ to FOXP3+ that is valuable in prognosis and prediction of SCC progression. They introduce immunotherapy as a revolution for HNSCC treatment. In our study, CD4+ TILs were lower with lymph involvement or distance metastasis in comparison to opposite groups, although this difference was not statistically significant, but it can be compared to this study. It seems small sample sizes impact our study. Moreover, they used advanced molecular methods such as microarray and ISH in addition to IHC that influenced the strength and value of the study.[45]

Recent approaches suggest next-generation profiling for TILs in cancers; this advanced technology helps us to figure out more about cancer immunopathology that noteworthy affects treatment outcome in patients. For example, immunoscore is an IHC-based assay that provides the signature of different types of TILs in TME. It represents two main profits: firstly, predict disease-free survival (DFS), and secondly, estimate tumor stage that facilitates tumor classification; it provides reliable prognosis and target candidate for immunotherapy.[25] This approach previously demonstrated impressive consensus in colorectal cancer.[46] The imaging mass cytometry is another way by phenotyping platform that evaluates multiple markers simultaneously. It is in early stage and is used considerably in clinical cancer research. These digital analysis technologies highlight the TIL subset role as prognostic and predictive biomarkers in tumor progression.[47]

Furthermore, in HNSCC patient's tumor mutation burden (TMB) influence CD4+ T-lymphocyte infiltration, and minimum TMB is better for predictive

prognosis of patients.[48] In this manner, although recent applicable immune therapeutic approaches for HNSCC patients are improved, reliable OS can better determine when the genetic variation of HNSCC patients is considered. Hence, we propose future studies can design by immune therapy base on TILs, and the genomic variation should be considered with advanced technology in HNSCC patients.

CONCLUSION

We can conclude that the significant differences of $CD4^+$ T-lymphocyte expression in healthy surgical margins compared to OSCC samples and in different grades demonstrated these cells involved in maturity, differentiation squamous cells, and disease progression. It seems that the healthy margins of tumor surgery are a valuable biological edge on for OSCC studies. And also, evaluation of similar tumor markers and the other associated molecular markers in margin can be effective in determination of safe margins. We suggest that $CD4^+$ TILs can apply for prognostic approaches.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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