Influencing Factors and Prognostic Significance of TIL Subset Abundance in Head and Neck Squamous Cell Carcinoma

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Research

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Abstract

Objective: To evaluate the abundance of Foxp3-positive tumor-infiltrating lymphocytes (Foxp3⁺TILs), CD103⁺cells, CD8⁺TILs, and CD3⁺TILs in tissue specimens of 85 head and neck squamous cell carcinoma (HNSCC) and analyze the associations of their abundance with the clinicopathological features and prognosis.

Methods: Immunohistochemistry was performed to detect the abundance of immunocyte subsets. Both correlation analysis and multivariate logistic regression analysis were used to analyze the associations between the frequency of these cell subsets and clinicopathological features in HNSCC. Kaplan–Meier analysis and the Cox proportional-hazards model were utilized to evaluate the effects of these immunocytes on the prognosis of patients with HNSCC.

Result: Multivariate logistic regression analysis showed that an independent association of Foxp3⁺TILs abundance with preoperative chemotherapy (PC) was negative ($P=0.018$), and that with smoking index ($P=0.001$) and N stage ($P=0.012$) was positive. Multivariate Cox model revealed that the high abundance of Foxp3⁺TILs was independently associated with short recurrence-free survival (RFS) ($P=0.015$) and poor overall survival (OS) ($P=0.015$). The high frequency of CD103⁺cells was independently related to long RFS ($P=0.001$) and favorable OS ($P<0.001$). The population with a high abundance of CD8⁺TILs and CD103⁺immunocytes in HNSCC tissues exhibited optimal RFS and OS, while the sub-group with a low frequency of CD8⁺TILs and CD103⁺immunocytes showed poor RFS and OS. Moreover, Spearman's correlation analysis revealed that the frequency of CD103⁺cells was positively correlated to the abundance of CD8⁺TILs in HNSCC ($P<0.001$).

Conclusion: Chemotherapy eliminates the frequency of Foxp3⁺TILs and improves the immune tumor microenvironment (iTME) of HNSCC. On the other hand, heavy smoking upregulates the abundance of Foxp3⁺TILs in HNSCC tissues and disrupt the balance of immune homeostasis in iTME. Additionally, both Foxp3⁺TILs and CD103⁺cells might be potential independent prognostic indicators of HNSCC.

1 Introduction

The immune tumor microenvironment (iTME) consists of several factors, such as immunocytes, which have a major influence on tumor progression. Recently, a number of studies have shown that tumor-infiltrating lymphocytes (TILs) play a vital role in the therapeutic response and prognosis of patients with solid tumors [1–8]. Typically, CD3 is expressed on almost all T cells, and CD3⁺TILs are heterogeneous and divided into CD4⁺TIL and CD8⁺TIL subsets, and the prognostic value of CD3⁺TILs in different solid tumors is controversial [4, 9]. CD8⁺TILs mainly produce a cytotoxic effect on cancer cells and is favorable to prognosis in solid tumor [4, 10]. However, CD8⁺TILs also show a larger transcriptional heterogeneity among individuals [11]. In addition, the bystanders in CD8⁺TILs do not respond to cancer cells [12]. Interestingly, several studies have proposed that CD103 is a marker of antigen-educated tumor-specific

CD8⁺TILs and maybe an independent and low-risk factor for predicting prognosis in solid tumors [5–8]. Previous studies have shown that forkhead box P3 (Foxp3) is a characteristic marker of regulatory T-cells (Tregs) [13, 14], and immunosuppressive Foxp3⁺Tregs are mainly CD4⁺TILs and is unfavorable to the prognosis of patients with cancer [15, 16]. Briefly, TILs are major elements in iTME and impact the prognosis of patients with solid tumors. A recent study has shown that preoperative chemotherapy (PC) improves the iTME *via* transforming the TILs subset landscape in breast cancer [2]. Nevertheless, the impact of the factors on the distribution of TIL subsets is yet to be investigated in depth. Therefore, immunohistochemistry (IHC) was performed to assay the abundance of TIL subsets (CD3, CD8, and Foxp3) and CD103⁺immunocytes in head and neck squamous cell carcinoma (HNSCC) and study the effect of clinicopathological factors on TIL landscape and the prognostic significance of these cell subsets in iTME.

2 Patients And Methods

2.1 Patients

A total of 85 patients who were histologically diagnosed with HNSCC between May 2012 and December 2013 at Tianjin Medical University Cancer Institute and Hospital (TMUCIH; Tianjin, China) were included in this study, and the focal information is described below, as reported in our previous studies [17, 18]. All the patients with HNSCC underwent radical surgery at TMUCIH. The inclusion criteria were as follows: (1) no radiotherapy before surgery, (2) pathological confirmation of the clinical diagnosis, and (3) providing sufficient samples to fulfill the requirements of experiments. The exclusion criteria were as follows: (1) patients with nasopharyngeal or oropharyngeal carcinoma, (2) patient's age < 16 or > 80-years-old, (3) no residual cancer cells on microscopic examination, or (4) missing follow-up and deaths unrelated to tumor progression. Pathological staging was estimated according to the 7th edition of the American Joint Committee on Cancer (AJCC) staging system for HNSCC. Follow-up was conducted on all the enrolled patients with HNSCC until December 2018. The median follow-up time was 61.7 (range, 3.9–67.6; mean, 48.6) months for patients who were alive at the last follow-up. During the follow-up period, 36 (36/85) patients had events of local recurrence or distant metastasis, and 34 patients died of HNSCC progression. Recurrence-free survival (RFS) was defined as the period from the date of radical surgery to the time of local recurrence or distant metastasis. Overall survival (OS) was defined as the duration from the date of radical surgery to death from cancer cause. Smoking index (SI) was defined as the number of cigarettes smoked daily multiplied by the total number of years of smoking. The demographic and clinicopathological features of these patients are summarized in Table 1.

2.2 IHC

The method of immunohistochemistry (IHC) was similar to that reported previously [17, 18]. Briefly, the formalin-fixed, paraffin-embedded (FFPE) tissue was sliced into 4-mm tissue sections on the microscope slides (Citotest, Shenzhen, China) and preheated at 70 °C for 1 h. After heat drying, the sections were deparaffinized in xylene and rehydrated in the gradient of ethanol. According to preset experimental

operation requirements, the citrate buffer (pH = 6.0) or ethylene diamine tetraacetic acid (pH = 9.0) was used for heat-induced epitope retrieval (HIER) at 123°C for 3 min, and the slides were slowly or immediately cooled for antigen retrieval. Subsequently, all the slides were immersed in 3% hydrogen peroxide for 20 min to inactivate the endogenous peroxidase of the specimens, followed by incubation with bovine serum albumin (BSA) to reduce the non-specific hydrophobic binding. Then, the tissue sections were incubated with mouse monoclonal primary antibodies against Foxp3 (SP97; dilution, 1:100; Abcam, CO, USA), CD103 (EPR4166(2); dilution, 1:500; Abcam), and CD3 (F7.2.38; dilution, 1:300; Thermo Fisher Scientific, Waltham, MA, USA), and rabbit monoclonal primary antibody against CD8 (SP16; dilution, 1:300; Thermo Fisher Scientific) at 4 °C overnight. After washing thrice with phosphate-buffered saline (PBS), the sections were incubated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Maixin Technology Co., Ltd, Shenzhen, China) for 30 min at room temperature before staining with 3,3'-diaminobenzidine (DAB). The color reaction was terminated by washing with deionized water. Finally, the slides were sealed with resin and mounted after dehydration in alcohol and xylene. The specimens were examined under a light microscope.

Two pathologists independently evaluated the IHC results of all samples. A total of ten high-power fields (HPF, × 400) were randomly selected at the strong density region of DAB-chromogenic cells, and the number of positive cells was recorded. The average number of positive cells was regarded as the abundance of DAB-chromogenic immunocytes. The receiver operating characteristic (ROC) curve was constructed using the method of minimum P-value in this cohort. $CD8^{+}TIL \geq 30/HPF$ was defined as $CD8^{+}TILs^{high}$ or the high abundance of $CD8^{+}TILs$, $CD8^{+}TIL < 30/HPF$ as $CD8^{+}TILs^{low}$ or the low abundance of $CD8^{+}TILs$, $CD3^{+}TIL \geq 59/HPF$ as $CD3^{+}TILs^{high}$ or the high frequency of $CD3^{+}TILs$ and $CD3^{+}TIL < 59/HPF$ as $CD3^{+}TILs^{low}$ or the low frequency of $CD3^{+}TILs$, $Foxp3^{+}TIL \geq 8/HPF$ as $Foxp3^{+}TILs^{high}$ or the high distributional level of $Foxp3^{+}TILs$ and $Foxp3^{+}TIL < 8/HPF$ as $Foxp3^{+}TILs^{low}$ or the low distributional level of $Foxp3^{+}TILs$, $CD103^{+}cells \geq 21/HPF$ as $CD103^{+}cells^{high}$ or the high abundance of $CD103^{+}cells$, and $CD103^{+}cells < 21/HPF$ as $CD103^{+}TILs^{low}$ or the low abundance of $CD103^{+}cells$.

2.3 Statistical analysis

Statistical analyses were performed using SPSS 24.0 software (IBM, Armonk, NY, USA). The chi-square test and Fisher's exact test were used to analyze the association between the abundance of immunocytes subsets ($CD3^{+}TIL$, $CD8^{+}TILs$, $CD103^{+}cells$, and $Foxp3^{+}TILs$) and clinicopathological features. The Spearman's correlation was applied to analyze the correlation among these subsets ($CD3^{+}TIL$, $CD8^{+}TILs$, and $CD103^{+}cells$). Mann-Whitney U test was utilized to analyze the correlation between smoking status and the abundance of $Foxp3^{+}TILs$ and to study the difference in the frequency of these subsets ($CD3^{+}TIL$, $CD8^{+}TILs$, $CD103^{+}cells$, and $Foxp3^{+}TILs$) between the PC and non-PC groups. Multivariate logistic regression analysis was used to screen the factors predicting the abundance of subsets ($CD8^{+}TILs$ and $Foxp3^{+}TILs$). RFS and OS data were estimated using the Kaplan–Meier method, and the log-rank test was used to compare the differences of high vs. low abundance of subsets ($CD3^{+}TIL$,

CD8⁺TILs, CD103⁺cells, and Foxp3⁺TILs). The Cox proportional-hazards model was used for multivariate regression analysis of independent prognostic factors associated with RFS/OS. The two-tailed $P < 0.05$ was considered statistically significant.

3 Results

3.1 Patients' clinical and demographic characteristics

A total of 85 HNSCC cases were enrolled in this study, as reported in previous investigations; also, the clinical and demographic characteristics of the patients were presented before [17, 18]. These patients with primary HNSCC included oral cancer ($n = 38$), laryngeal cancer ($n = 36$), hypopharyngeal cancer ($n = 9$), as well as other cancers ($n = 2$). The constituent ratio of patients at pathological stages I, II, III, and IV was 36.5%, 18.8%, 15.3%, and 29.4%, respectively. Moreover, 21 cancer tissues contained poorly differentiated clusters (PDCs). The age of the patients was 27–76 (median age, 57)-years-old, and the cohort consisted of 18 (22.2%) female cases, 49 (57.6%) with alcohol consumption history, 54 (63.5%) with the history of smoking, and 54 (63.5%), who received chemotherapy before radical surgery. In addition, the baseline characteristics of Foxp3⁺TILs abundance in patients with HNSCC are listed in Table 1, and those of other immunocyte subsets are presented in Suppl. tables

3.2 Staining and abundance of immunocyte subsets in HNSCC

The staining characteristics of CD3, CD8, CD103, and Foxp3 are illustrated in the sections of FFPE tissue from four patients (Fig. 1). The IHC of CD3 and CD8 showed yellow staining in the membrane of TILs, and the coloration of CD103 was also in the membrane of cells, which was infiltrating in carcinoma tissues; however, Foxp3 staining exhibited in the nucleus of TILs. Among 85 patients with HNSCC, 39 cases (45.9%) showed the abundance of CD3⁺TILs < 59 /HPF, 46 cases (54.1%) exhibited the frequency of CD3⁺TILs ≥ 59 /HPF, 26 cases (30.6%) presented the frequency of CD3⁺TILs < 30 /HPF, 59 cases (69.4%) emerged the abundance of CD3⁺TILs ≥ 30 /HPF, 39 cases (45.9%) showed the abundance of CD103⁺cells < 21 /HPF, 46 cases (54.1%) exhibited the frequency of CD103⁺cells ≥ 21 /HPF, 44 cases (51.8%) presented the frequency of Foxp3⁺TILs < 8 /HPF, and 41 cases (48.2%) emerged the abundance of Foxp3⁺TILs ≥ 8 /HPF. Furthermore, among 59 patients with high abundance of CD8⁺TILs, 39 cases (66.1%) exhibited CD103⁺cells^{high} and 20 cases (33.9%) displayed CD103⁺cells^{low}, while 26 patients showed low abundance of CD8⁺TILs, 7 cases (26.9%) had CD103⁺cells^{high}, and 19 cases (73.1%) presented CD103⁺cells^{low}.

3.3 Correlation between immunocyte subset abundance and clinicopathological features

In the current study, we found that a significantly high abundance of Foxp3⁺TILs was correlated to gender, site of primary cancer, and smoking history (Table 1). A significant increase was detected in the

incidence of the high frequency of Foxp3⁺TILs with male gender, history of smoking (SI $\geq$ 1000), and primary laryngeal carcinoma, i.e., 22.2% (4/18) and 55.2% (37/67) in female cases vs. male cases (P = 0.017), 40.0% (26/65) and 75.0% (15/20) from cases with IS < 1000 vs. IS $\geq$ 1000 (P = 0.006), and 36.7% (18/49) and 63.9% (23/36) from cases with non-laryngeal carcinoma vs. primary laryngeal carcinoma (P = 0.013). However, an obvious decrease was noted in the incidence of a high abundance of Foxp3⁺TILs in cases with oral cancer, i.e., 63.8% (30/47) and 28.9% (11/38) from the cases with non-oral carcinoma vs. primary oral carcinoma (P = 0.001). The results of the Mann–Whitney U test revealed that PC and smoking history were significantly associated with the abundance of Foxp3⁺TILs (Fig. 2). However, no evident association was detected between the frequency of Foxp3⁺TILs and age, laryngopharynx, stages (T, N, and TNM), PDCs, or drinking. Additionally, we performed multivariate logistic regression analysis, and it was disclosed that the abundance of Foxp3⁺TILs was independently associated with PC, SI, and N stage (Table 2).

We also showed that a significantly higher frequency of CD8⁺TILs was related to the sites of primary cancer, local lymph node metastasis, and the sample mixed PDCs (Suppl. Table 2). A significant decrease was noted in the incidence of the high abundance of CD8⁺TILs with primary hypopharyngeal carcinoma, lymph node metastasis (N1–N3), and mixed PDCs, i.e., 73.7% (56/76) and 33.3% (3/9) from cases with non-hypopharyngeal carcinoma vs. primary hypopharyngeal carcinoma (P = 0.021), 79.7% (47/59) and 46.2% (12/26) from cases with N0 stage vs. N1–N3 stage (P = 0.003), and 80.3% (49/61) and 47.6% (10/21) from samples with PDCs mixed vs. PDCs absent (P = 0.004). However, an obvious increase was observed in the incidence of the high abundance of Foxp3⁺TILs with oral cancer, i.e., 59.6% (28/47) and 81.6% (31/38) from cases with non-oral carcinoma vs. primary oral carcinoma (P = 0.029). Also, no correlation was established between the frequency of CD8⁺TILs and age, gender, larynx, stages (T and TNM), PC, smoking, or drinking. Moreover, multivariate logistic regression analysis was performed, which disclosed that the abundance of CD8⁺TILs was independently associated with the primary sites of cancer (oral carcinoma) (Suppl. Table 3).

Furthermore, no correlation was found between the clinicopathological factors mentioned above and the CD3⁺TIL abundance or CD103⁺cells frequency (Suppl. Table 1, Suppl. Table 4, and Fig. 2E). Subsequently, the Spearman's correlation analysis discovered a positive correlation was between CD3⁺TILs frequency and CD8⁺TIL abundance, CD3⁺TIL abundance and CD103⁺cell frequency, and CD8⁺TIL frequency and CD103⁺cell abundance (Fig. 2), rather than between Foxp3⁺TIL frequency and CD3⁺TILs, CD8⁺TILs, or CD103⁺cells (data not shown).

3.4 Associations of immunocytes subset abundance with prognoses in HNSCC patients

The results of Kaplan–Meier analysis and log-rank test revealed that both CD8⁺TILs^{high} and CD103⁺cells^{high} were markedly correlated with long RFS (P < 0.001 and P = 0.002, respectively) and favorable OS (all P = 0.001) rather than CD3⁺TIL^{high}; however, Foxp3⁺TILs^{high} was obviously associated

with short RFS ($P = 0.001$) and poor OS ($P = 0.004$), respectively (Fig. 3). The Cox proportional-hazards regression model was utilized to identify the effects of clinicopathological prognostic factors on the survival of patients with HNSCC. Univariate Cox model analysis demonstrated that the sites of primary cancer (hypopharyngeal carcinoma), pathological stages (T, N, and TNM), degree of differentiation, and the frequency of Foxp3⁺TILs were correlated with short RFS of patients with HNSCC (all $P < 0.05$; Table 3). In addition, the sites of primary cancer (hypopharyngeal carcinoma), pathological stages (T, N, and TNM), and the frequency of Foxp3⁺TILs were correlated with poor OS of patients with HNSCC (all $P < 0.05$; Table 4) rather than the degree of differentiation. However, CD8⁺TILs^{high} and CD103⁺cells^{high} were associated with long RFS and favorable OS (all $P < 0.05$; Tables 3 and 4). Multivariate Cox model revealed that the high abundance of Foxp3⁺TILs was independently associated with short RFS (hazard ratio (HR) = 3.014, 95% confidence interval (CI): 1.236–7.348; $P = 0.015$; Table 3) and poor OS (HR = 2.802, 95% CI: 1.226–6.399; $P = 0.015$; Table 4). The high frequency of CD103⁺cells was independently related to long RFS (HR = 0.300, 95% CI: 0.145–0.620; $P = 0.001$; Table 3) and improved OS (HR = 0.253, 95% CI: 0.117–0.546; $P < 0.001$; Table 4) rather than CD8⁺TILs^{high}. Especially, the sub-population with a high abundance of CD8⁺TILs and CD103⁺immunocytes in HNSCC tissues had the best RFS and OS, while the sub-group with a low frequency of CD8⁺TILs and CD103⁺immunocytes had the worst RFS and OS (Fig. 4).

4 Discussion

In the treatment of HNSCC, the recommendation for induction chemotherapy (IC) is commonly based on the update from RTOG 91 – 11 [19]. Several phase III trials showed significantly improved survival for patients in the 3-drug induction group (cisplatin, 5-FU plus taxane; TPF) as compared to those receiving 2 drugs (cisplatin plus 5-FU) [20–24]; this result was supported by a meta-analysis encompassing five randomized controlled trials (RCTs) [25]. Due to the adjustment of treatment strategy, after IC, some patients with HNSCC underwent radical surgery, instead of concurrent chemoradiation, which provided an advantage in understanding the transformation of iTME after chemotherapy for HNSCC.

In these patients with HNSCC, Foxp3⁺TILs was significantly eliminated in the PC sub-group as compared to the non-PC sub-population, rather than CD3⁺TILs, CD8⁺TILs, and CD103⁺cells. Docetaxel is a microtubule inhibitor and should preferentially inhibit the proliferation and function of rapidly dividing Tregs, instead of CD4⁺ effector T cells [26], in which support our finding that Foxp3⁺TILs are eliminated. In addition, several preclinical trials have shown that docetaxel also enhances CD8⁺T-cell responses and antitumor activity [27, 28], although our results showed that the frequency of CD8⁺TILs and CD103⁺cells in the sub-group that received PC was not decreased significantly than that in the non-PC sub-population. Briefly, PC may be the main reason for the transformation of the T-cell landscape, as substantiated previously [29, 30].

Smoking, drinking, chewing betel nut, and virus infections play a pivotal role in the progression and development of HNSCC. The smoking-related carcinogen, 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) elevates the expression of HIF-1 α and HIF-2 α [31]. HIF-1 α directly binds to the Foxp3 promoter and upregulates Foxp3 expression in the cutaneous T cells [32]. The result of multivariate

logistic regression analysis disclosed that the abundance of Foxp3⁺TILs was independently associated with the smoking history of the patients with HNSCC. These results indicated that smoking carcinogen, such as NNK, upregulates the abundance of Foxp3⁺TILs in HNSCC *via* HIF-1 signaling pathway.

Additionally, the result of multivariate Cox regression analysis showed that the high frequency of Foxp3⁺TILs is one of the independent risk factors for the survival of the patients with HNSCC. Several studies have shown that Foxp3 is a characteristic marker of Tregs [13, 14], and immunosuppressive Foxp3⁺Tregs are unfavorable to the prognosis of patients with solid tumors [15, 16]. Consequently, the result of the current study demonstrated that Foxp3⁺TIL in HNSCC might be a dominant subset of Tregs with immunosuppression.

Herein, the results of survival analysis established a correlation between CD8⁺TILs^{high}/CD103⁺cells^{high} and long RFS and optimal OS in patients with HNSCC. The Spearman's correlation analysis demonstrated that the frequency of CD8⁺TILs was positively correlated with the abundance of CD103⁺cells, and multivariate Cox regression analyses revealed that CD103⁺cells^{high} was deemed as one of the independent factors for predicting favorable survival rather than CD8⁺TILs^{high}. Moreover, the combined analysis of the effects of CD8⁺TILs^{high} and CD103⁺cells^{high} on the survival of patients with HNSCC revealed maximal RFS and OS for the sub-population with a high abundance of CD8⁺TILs and CD103⁺immunocytes in HNSCC tissues. Several studies also showed that the frequency of CD103⁺CD8⁺TILs was associated with the level of granzyme B, which is critical to kill cancer cells, and the CD8⁺CD103⁺TILs or CD103⁺TILs are favorable to the prognosis in breast cancer, lung cancer, and laryngeal squamous cell carcinoma [5, 8, 33, 34]. These findings suggested that CD103 is a vital biomarker of cytotoxic CD8⁺TILs in solid tumors, while the frequency of CD103⁺cells may be an indicator for the prognosis of some solid tumors.

Interestingly, the abundance of CD8⁺TILs in oral carcinoma tissues was higher than that in the HNSCC originating from other sites, and the association of original site of the tumor with the frequency of CD8⁺TILs was independent. Nevertheless, the independent and positive association of CD8⁺TILs^{high} with oral carcinoma needs to be elucidated with respect to the mechanisms underlying this phenomenon. Since the comprehensive knowledge of the mechanism is yet lacking, we speculate that the phenomenon may be important when considering the trial of immunotherapy strategies for HNSCC.

5. Conclusions

Chemotherapy significantly eliminates the frequency of Foxp3⁺TILs and improves the iTME of HNSCC. Conversely, heavy smoking upregulates the abundance of Foxp3⁺TILs in HNSCC tissues and overturn the balance of immune homeostasis in iTME. Additionally, both Foxp3⁺TILs and CD103⁺cells may be potentially independent prognostic indicators of HNSCC.

Abbreviations

DAB: 3,3'-diaminobenzidine

Foxp3: forkhead box P3

FFPE: formalin-fixed, paraffin-embedded

HNSCC: head and neck squamous cell carcinoma

HPF: high-power fields

iTME: immune tumor microenvironment

IHC: immunohistochemistry

IC: induction chemotherapy

NNK: 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone

OS: overall survival

PC: preoperative chemotherapy

PDCs: poorly differentiated clusters

RFS: recurrence-free survival

SI: smoking index

TILs: tumor-infiltrating lymphocytes

Tregs: regulatory T-cells

Declarations

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to them being collected within the institution but are available from the corresponding author on reasonable request.

Authors' contributions

Xinwei Zhang and Li Zhou contributed to the conception and design of the study. Preparation of materials, performing the experiments, data collection, and data analysis were conducted by Xuezhou

Wang Li Zhou, Ruifeng Dong, Baihui Li, and Wenwen Yu. The first draft of the manuscript was prepared by Xuezhou Wang. All the authors read the first version of the manuscript and provided their valuable comments, and Xinwei Zhang and Xiubao Ren revised the manuscript accordingly. All the authors read and approved the final version of the manuscript.

Ethics statement

The present study was approved by the Ethics Committee of TMUCH, and the study was conducted in accordance with the Declaration of Helsinki. Patients or their guardians have given their written informed consent.

Consent for publication

We just extracted data and did not involve the private information of patients.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Tables

Table 1 Patient characteristics and association between the abundance of Foxp3⁺TILs and clinicopathological factors

Characteristic	n	Foxp3 ⁺ TILs		P
		low	high	
Gender				0.017*
Male	67	30	37	
Female	18	14	4	
Age (years)				0.478
< 60	51	28	23	
≥ 60	34	16	18	
Sites 1				0.082*
Non-laryngopharynx	76	42	34	
Laryngopharynx	9	2	7	
Sites 2				0.001
Non-oral cavity	47	17	30	
Oral cavity	38	27	11	
Sites 3				0.013
Non-larynx	49	31	18	
Larynx	36	13	23	
T stage				0.350
T1	35	16	19	
T2–T4	50	28	22	
N stage				0.103
N0	59	34	25	
N1–N3	26	10	16	
TNM stage				0.169
I	31	13	18	
II–III	54	31	23	
Poor differentiation				
Absent	61	35	26	0.057
Mixed	21	7	14	

Smoking index				0.006
< 1000	65	39	26	
≥ 1000	20	5	15	
Drinking				0.299
Non and mild	49	21	15	
Constant	36	23	26	
Preoperative chemotherapy				0.169
Absent	31	13	18	
Received	54	31	23	
CD3 ⁺ TILs				0.165
Low	39	17	22	
High	46	27	19	
CD8 ⁺ TILs				0.247
Low	26	11	15	
High	59	33	26	
CD103 ⁺ cells				0.430
Low	39	22	17	
High	46	22	24	

* Fisher's exact test

Table 2 Multivariate logistic regression analysis of the association between the abundance of Foxp3⁺TILs and clinicopathological factors

Factors	β	Wald χ^2	Exp (B) (95% CI)	P
Gender (male vs. female)	-0.557	0.674	0.573 (0.151–2.166)	0.412
Sites 1 (Non-laryngopharynx vs. laryngopharynx)	1.064	1.304	2.897 (0.467–17.992)	0.254
Sites 2 (Non-oral cavity vs. oral cavity)	0.872	1.135	2.391 (0.481–11.879)	0.287
Sites 3 (Non-larynx vs. larynx)	0.761	1.990	2.140 (0.744–6.158)	0.158
N stage (N0 vs. N1–N3)	1.438	6.288	4.212 (1.369–12.958)	0.012
TNM stage (I vs. II–III)	-0.726	0.636	0.484 (0.139–1.684)	0.254
Poor differentiation (absent vs. mixed)	0.553	3.896	1.739 (0.431–7.010)	0.437
Smoking index (< 1000 vs. $\geq$ 1000)	2.444	11.946	11.515 (2.880–46.033)	0.001
Preoperative chemotherapy (absent vs. received)	-1.099	5.640	0.333 (0.135–0.825)	0.018
CD3 ⁺ TILs (low vs. high)	-0.712	2.801	0.491 (0.213–1.130)	0.094

Table 3 Univariate and multivariate Cox regression analyses of clinicopathological factors related to recurrence-free survival

Factors	Univariate regression analysis		Multivariate regression analysis	
	HR (95% CI)	P	HR (95% CI)	P
Gender				
(male vs. female)	0.500 (0.194–1.287)	0.151	0.437 (0.161–1.185)	0.104
Age (years)				
< 60 vs. ≥ 60	1.086 (0.560–2.107)	0.807	-	-
Sites 1				
Non-laryngopharynx vs. laryngopharynx	3.359 (1.529–7.539)	0.003	1.032 (0.369–2.885)	0.952
Sites 2				
Non-oral cavity vs. oral cavity	0.642 (0.325–1.268)	0.202	-	-
Sites 3				
Non-larynx vs. larynx	0.824 (0.421–1.610)	0.570	-	-
T stage				
T1 vs. T2–T4	3.438 (1.564–7.588)	0.002	2.841 (1.109–7.281)	0.030
N stage				
N0 vs. N1–N3	4.021 (2.077–7.784)	0.000	2.508 (1.013–6.207)	0.047
TNM stage				
Ⅰ vs. Ⅱ–Ⅲ	2.682 (1.221–5.891)	0.014	1.413 (0.545–3.663)	0.477
Poor differentiation				
Absent vs. mixed	2.088 (1.036–4.207)	0.039	1.184 (0.533–2.630)	0.679
Smoking index				
< 1000 vs. ≥ 1000	1.393 (0.672–2.890)	0.373	-	-
Drinking				
No and mild vs. constant	1.517 (0.789–	0.211	-	-

	2.916)			
Preoperative chemotherapy				
Absent vs. received	1.382 (0.691–2.765)	0.360	-	-
CD3 ⁺ TILs				
Low vs. high	0.549 (0.283–1.066)	0.077	0.870 (0.414–1.831)	0.715
CD8 ⁺ TILs				
Low vs. high	0.311 (0.161–0.601)	0.001	1.251 (0.480–3.258)	0.647
CD103 ⁺ Cells				
Low vs. high	0.362 (0.183–0.717)	0.004	0.300 (0.145–0.620)	0.001
Foxp3 ⁺ TILs				
Low vs. high	3.098 (1.518–6.319)	0.002	3.014 (1.236–7.348)	0.015

Table 4 Univariate and multivariate Cox regression analyses of clinicopathological factors related to overall survival

Factors	Univariate regression analysis	Multivariate regression analysis		
	HR (95% CI)	P	HR (95% CI)	P
Gender				
(male vs. female)	0.562 (0.217–1.451)	0.234	-	-
Age (years)				
< 60 vs. ≥ 60	1.081 (0.546–2.141)	0.822	-	-
Sites				
Non-laryngopharynx vs. laryngopharynx	4.361 (1.942–9.794)	0.000	1.737 (0.690–4.371)	0.241
Sites 2				
Non-oral cavity vs. oral cavity	0.716 (0.358–1.430)	0.343	-	-
Sites 3				
Non-larynx vs. larynx	0.685 (0.339–1.385)	0.292	-	-
T stage				
T1 vs. T2–T4	3.636 (1.581–8.363)	0.002	2.239 (0.865–5.796)	0.097
N stage				
N0 vs. N1~N3	4.062 (2.061–8.007)	0.000	2.960 (1.250–7.005)	0.014
TNM stage				
Ⅰ vs. Ⅱ–Ⅲ	2.844 (1.237–6.537)	0.014	1.532 (0.571–4.115)	0.397
Poor differentiation				
Absent vs. mixed	2.054 (0.995–4.240)	0.052	0.942 (0.382–2.323)	0.896
Smoking index				
< 1000 vs. ≥ 1000	1.226 (0.572–2.627)	0.601	-	-
Drinking				
No and mild vs. constant	1.242 (0.633–	0.528	-	-

	2.436)			
Preoperative chemotherapy				
Absent vs. received	1.179 (0.584–2.383)	0.646	-	-
CD3 ⁺ TILs				
Low vs. high	0.602 (0.306–1.186)	0.142	0.907 (0.434–1.894)	0.794
CD8 ⁺ TILs				
Low vs. high	0.336 (0.170–0.661)	0.002	1.160 (0.446–3.019)	0.761
CD103 ⁺ Cells				
Low vs. high	0.313 (0.152–0.645)	0.002	0.253 (0.117–0.546)	0.000
Foxp3 ⁺ TILs				
Low vs. high	2.732 (1.330–5.610)	0.006	2.802 (1.226–6.399)	0.015

Figures

Figure 1

Immunohistochemical staining Abundance of immunocyte subsets in head and neck squamous cell carcinoma tissues under high-magnification (400×): re-pathological diagnosis of serial tumor tissue sections (A, B, C, and D correspond to E, F, G, and H, respectively), and CD3+TILs (E), CD8+TILs (F), CD103+cells (G), and Foxp3+TILs (H).

Figure 2

Correlation analysis between variables and Mann-Whitney U test of TILs frequency Association of CD3⁺TILs abundance with CD8⁺TILs frequency (A), CD3⁺TILs abundance with CD103⁺cells frequency (B), CD8⁺TILs abundance with CD103⁺cells frequency (C), smoking index (SI, counts of daily smoking × years) with Fxp3⁺TILs frequency (D), and preoperative chemotherapy (PC) with immunocyte subsets abundance (E) in head and neck squamous cell carcinoma. ***, $P < 0.001$; *, $P < 0.05$.

Figure 3

Kaplan–Meier survival analysis Recurrence-free survival and overall survival curves of the HNSCC patients with high vs. low CD3+TILs (A and E), CD8+TILs (B and F), CD103+cells (C and G), and Foxp3+TILs (D and H) abundance.

Figure 4

Combination survival analysis Recurrence-free survival and overall survival curves of the HNSCC patients with high vs. low CD8+TILs and CD103+cell frequency.

Supplementary Files

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