The role of tumor infiltrating lymphocytes (TILs) and the ratios between different subsets serve as prognostic factors in hypopharyngeal squamous cell carcinoma

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Abstract

**Background:** Cancer cells induce the infiltration of various immune cells located or distributed in different sites and playing multiple roles, which have recently been proposed to predict clinical outcomes. We therefore studied the prognostic significance based on the presence of tumor infiltrating lymphocytes (TILs) and the ratios between different types of immune cells in hypopharyngeal squamous cell carcinoma (HPSCC).

**Methods:** We retrospectively analysed 132 consecutive patients diagnosed with HPSCC in 2013-2017. Tumoral parenchyma were immunohistochemically counted manually for the number of CD8, CD4 and Foxp3. The ratios of CD8/Foxp3 and CD4/CD8 were calculated for each specimen, and analyzed with respect to patient clinicopathologic variables and prognosis.

**Results:** HPSCC patients with high levels of TILs showed evidently correlations with well differentiated tumors (P < 0.05). Increased Foxp3+ TIL is also significantly associated with Stage and T stage (P = 0.048 and P = 0.046, respectively). Kaplan-Meier analysis showed that high CD8 and FoxP3 infiltration correlated with favorable overall survival (OS, P = 0.019 and P = 0.001), disease-free survival (DFS, P = 0.045 and P = 0.028) and distant metastasis-free survival (DMFS, P = 0.034 and P = 0.009), respectively, but only FoxP3 displayed prognostic significance for DMFS in multivariate analysis (MVA). In lymphocyte ratios analysis, CD8/FoxP3 appeared to play a pivotal role, patients with high CD8/FoxP3 ratio had a superior 3-year DFS and DMFS compared with the low value of this parameter in both univariate analysis (UVA) and MVA (P = 0.015 and P = 0.001). Meanwhile, high CD4/CD8 ratio had significantly better DFS and local relapse-free survival (LRFS) in UVA, and exhibited an independent prognostic factor for improved LRFS in MVA (P = 0.040).

**Conclusion:** Although high TILs were determined to be prognostically significant in HPSCC, the ratios of these subsets may be more informative. Particularly, higher ratio of CD8/Foxp3 accurately predict prognosis for improved DFS and DMFS, and increment of CD4/CD8 ratio was an independent predictor for favorable LRFS.

**Background**

Hypopharyngeal squamous cell carcinoma (HPSCC) is a highly malignant disease of head and neck
cancer, which is the eighth common cancer worldwide [1]. Although the incidence is comparatively low, HPSCC is usually diagnosed at an advanced stage for unapparent early symptom [2]. Although there are many treatments, such as surgery, concurrent chemoradiation therapy (CCRT) or radiation therapy, the five-year survival rate is less than 35% [3, 4]. Given the difficulty of diagnosing HPSCC at an early stage as well as its severe prognosis, new approaches concerning prognostic evaluation and treatment alternatives are necessary. It is urgently to find novel biological factors that accurately predict clinical outcomes for HPSCC patients. 

Recent years it has been increasingly recognized that the immune microenvironment is the “battle field” between tumor progression and the immune system defense. Immune surveillance and immune escape provide a dynamic balance, inhibiting tumor progression by recognizing and killing tumor cells, and weakening the anti-tumor activity of immune cells by expressing inhibitory molecules and secreting cytokines [5]. Tumor-infiltrating lymphocytes (TILs), as an important actor in the tumor immune microenvironment, is a heterogeneous lymphocyte population mainly composed of T lymphocytes, which was first proposed in 1986 and has been proved to be an independent prognostic biomarker in various tumors [6–9]. Growing evidence indicated that TILs consist of numerous anti-tumor effector or regulatory T cells (Tregs) and serve as key players in a host’s immune response to tumor. Thus, evaluating the functions of different TIL subsets are thought to be better understanding of tumor progression and effective anti-tumor strategies. In fact, the most consistently beneficial TILs seem to be CD8 + TILs, which regarded as cytotoxic T lymphocytes (CTLs), and specifically recognize and destroy target cells [10]. It has been reported to be the major effector cells for tumor elimination by recognizing tumor-derived antigenic epitopes [11]. On the contrary, Foxp3 + TILs have been classified as Tregs, and may actually contribute to suppressing anti-tumour immune responses [12]. In most studies, Tregs are generally considered to play a crucial role in the process of immune escape, helping tumor cells avoid from immunological surveillance. However, it is also true that the prognostic significance of Foxp3 + TILs remains controversial. For instance, Foxp3 + TIL was reported to be linked to favorable clinical outcomes in non-small cell lung cancer (NSCLC), sinonasal squamous cell carcinoma and so on [13, 14], but others supported that Foxp3 + TIL correlated to worse
prognosis [15, 16]. Furthermore, CD4 + TILs are derived from T cells mediated by IL-2, which contain T helper cell population and regulatory T cells (Tregs). In terms of anti-tumour immunity, T helper cells activation are effective and play an important role in inducing or motivating CTLs, whereas CD4 + Tregs suppress effector T lymphocytes [17, 18]. However, whether these pro-tumor effects outweigh anti-tumor effects or are equal in a particular tumor is debatable. This could explain why the benefits of CD4 + T cell infiltration on the prognosis of different tumors are somewhat inconsistent. From the above, it is evident that TILs may act as a double-edged sword, and the relations between the different types of immune cells have not been thoroughly examined. More recently, the hypothesis that lymphocyte ratios could have more prognostic significance has gained much attention. Emerging evidence showed that higher ratios of CD8+/Foxp3 + and CD4/CD8 are more sensitive indicators for prognosis and for monitoring immune function, even acting as biomarkers to predict tumor relapse and responses to treatment [13, 19–21]. A study by Sideras et al examined fresh metastatic tissues of 47 patients with colorectal cancer liver metastases, also illustrating that high CD8+/FoxP3 + ratio was found to be an independent predictor of survival [22]. Specifically, ratios of these subsets may provide a more comprehensive view of what occurs in the tumor microenvironment and which of T cell subtype dominates or is likely to overshadow the functions of other T-cells. Previous works have demonstrated that high CD8 and Foxp3 expression contributed to better overall survival (OS) and disease-free survival (DFS) in HPSCC, yet the correlations of CD8/Foxp3 and CD4/CD8 to clinical outcomes remain unclear.

Based on the consideration that the quantitative ratios are probably more important in the tumor immune microenvironment. This study focuses on the prognostic meaning of TILs and the ratios of CD8/Foxp3 and CD4/CD8 in relation to clinical outcomes, and further seeks to determine more reliable biomarkers in a relatively larger HPSCC cohort, which may appropriate select out high-risk patients eligible for more aggressive therapeutic agents.

Methods

**Specimens and patients**

The present study enrolled 132 patients with HPSCC from 2013 to 2017, who underwent surgical
treatment at the Eye and ENT Hospital of Fudan University, Shanghai, China. No patients received neoadjuvant chemotherapy or other therapies. All HPSCC specimens were fixed in 10% formalin and embedded in paraffin for histopathological analysis and immunohistochemistry uses. Hematoxylin-eosin (HE) staining of sections was performed in automated stainer/coverslipper workstation (Leica HistoCore SPECTRA ST). Complete clinical data were collected and all patients gave written informed consent before surgery. The Institutional Review Committee of the Eye and ENT Hospital granted ethical approval.

We used four clinical end points in this study: 1) overall survival (OS) was defined as the time from surgery until the date of death from any cause, 2) disease-free survival (DFS) was defined as the time from surgery until the date of first recurrence/metastasis or death from any cause, 3) distant metastasis-free survival (DMFS) was defined as the time from surgery until the date of distant metastasis of the tumor or occurrence of death from any cause, and 4) local relapse-free survival (LRFS) was defined as the time from surgery until the date of local recurrence or the death from any cause.

**Immunohistochemical (IHC) staining and evaluation**

Immunohistochemical staining was performed in automated immunostainer (Ventana Medical System, USA) using three antibodies: CD8 (SP16, Gene Tech, Shanghai, China), CD4 (EP204, Gene Tech, Shanghai, China) and Foxp3 (98377, CST, Danvers, MA, USA). Sections of 4mm were placed on glue-coated glass slides (PRO-01, Matsunami, Japan). Human tonsil sections were used as positive controls for CD8, Foxp3 and CD4. Negative control was performed by omitting the primary antibody. All conditions and procedures were defined as the same as our previous studies.[23] Tumoral parenchyma (tumor bed) was distinguished from the stroma using HE staining and the levels of CD8, Foxp3 and CD4 expression were counted manually under 10 randomly high-power fields (400X) for each slide.

**Statistical analysis**

Statistical analyses were performed by using SPSS (22.0, IBM, Armonk, NY, USA). Fisher’s exact test and the chi-squared tests were used to evaluate the associations among the variables. The
relationships between the different lymphocyte infiltrates were calculated using Pearson’s correlation coefficient. The Kaplan-Meier method and log-rank test were conducted to determine the prognosis at different survival end points. Univariate and multivariate analyses (UVA and MVA) of prognostic factors were performed using the Cox proportional hazards model. P < 0.05 was considered statistically significant.

Results

Patient characteristics

A total of 132 consecutive patients in this cohort were diagnosed with HPSCC and the clinical characteristics of these patients are summarized in Table 1. The samples included 131 males and 1 female with a median age of 60 years (range: 40-76 years). A higher pathological grading (Grade III) group numbered 29 (22%), and the lower gradings (Grades I and II) group accounted for 103 (78%) patients. As described above, patients were divided into 2 groups depending on HPSCC clinical stage according to the AJCC 7th edition cancer staging system: namely Stage III (35, patients, 26.5%) and Stage IVA or IVB (97 patients, 73.5%). Patients smoked at least 20 packs of cigarettes per year as many as 115 (87.1%) and smoking less than 20 packs group was 17 (12.9%). Most tumors were located in the pyriform sinus (PS).

Follow up

With a median follow-up of 28.4 months (Interquartile Range 20.9-39.1 months), the 3-year OS, DFS, DMFS and LRFS for the entire cohort were 68.2% (95% confidence interval [CI], 57.8% to 78.6%), 62.1% (95%CI, 52.1% to 72.1%), 72.6% (95%CI, 62.2% to 83.0%) and 79.7% (95%CI, 72.4% to 87.0%), respectively. During the follow-up period, treatment failed in 42 (31.8%) patients. Local-regional recurrence was observed in 25 (18.9%) patients, and distant metastasis occurred in 26 (19.7%) patients.

Expression of CD8, CD4 and Foxp3

Representative images for immunohistochemical detection of tumor-infiltrating T lymphocytes are shown (Fig. 1A-F). Two independent pathologist who were blinded to the patient data reviewed the slides. Median values were used for cut-offs and the patient cohort was separated into high and low
groups, as described in our previous study. The medians were 80 for CD8 (range 1 to 900), 30 for Foxp3 (range 2 to 300) and 30 for CD4 (range 1 to 400), respectively. Areas of the tumor with hemorrhage or necrosis were avoided. We also investigated the ratios of CD8/Foxp3 and CD4/CD8, calculating them for each individual tumor. Similarly, the optimal cut-off points were calculated, along with their medians: the values were 2.50 (range 0.1 to 33.33) for CD8/Foxp3 and 0.33 (range 0.01 to 6.00) for CD4/CD8.

**Association among different variables**

Regarding the correlation of the immune markers with the clinicopathologic characteristics, high levels of TILs (CD8, Foxp3 and CD4) showed evidently correlations with lower histopathological grade and the ratios of CD8/Foxp3 and CD4/CD8 were correlated with the expression of each subtype (CD8, Foxp3 and CD4, CD8, respectively) (P < 0.05). Increased Foxp3+ TIL also exhibited a significant association with both stage and T stage (P = 0.048 and P = 0.046, respectively). We also found markedly correlations among CD8, CD4 and FoxP3, using Pearson’s correlation coefficient (P < 0.001, Fig. 2A-C). Other relationships between immune markers expression and clinicopathologic parameters are summarized in Table1.

**Correlation with prognosis**

The Kaplan-Meier curves of 3-year OS, DFS, DMFS, LRFS for patients with TILs and the ratios (low or high) are shown (Figs. 3-4). The 3-year OS, DFS, DMFS and LRFS rates, according to high and low CD8+TIL density, were 80.9% vs 56.3%, 73.2% vs 51.4%, 80.4% vs 64.5% and 77.8% vs 82.1%, respectively. Significant differences were found between the high and low CD8+TIL groups in 3-year OS, DFS and DMFS, but not in the LRFS (Fig. 3A-D). Similarly, a higher FOXP3+TIL was also strongly correlated with better OS, DFS and DMFS (P = 0.001, P = 0.028 and P = 0.009, respectively, Fig. 3E-H). Further analysis revealed that patients with a high CD8/FoxP3 ratio had significantly better DFS and DMFS (P = 0.013 and P = 0.029, respectively) (Fig. 4B-C), while higher ratio of CD4/CD8 had evidently improved 3-year DFS and LRFS compared with lower CD4/CD8 ratio (P = 0.029 and P = 0.033, respectively) (Fig. 4F, H). By contrast, no association between the status of CD8/FoxP3 or CD4/CD8 ratio and OS was observed (Fig. 4A, E). Both UVA and MVA were performed to determine the
associations between prognosis and clinic-pathological variables (Table 2-3). The multivariate variables were adopted from their prognostic significance in UVA (P < 0.05). The results did reveal that high ratio of CD8/FoxP3 remained an independent favorable prognostic factor for DFS (HR=2.613; 95% CI, 1.203-5.673; P =0.015) and DMFS (HR=3.606; 95% CI, 1.334-9.748; P = 0.011). Meanwhile, the ratio of CD4/CD8 was also an independent prognostic factor for LRFS (HR=0.414; 95% CI,0.178-0.959; P = 0.040) in the MVA. In addition, FoxP3+ TIL, T stage and site were found to be independent prognosis factors associated with DMFS, DFS and LRFS, respectively (Table 3).

Discussion

Our study is the first to evaluate lymphocyte ratios in HPSCC and their correlations to clinicopathological characteristics and prognosis in more than 100 patients with surgery. The results indicated that high ratio of CD8/Foxp3 accurately predict improved prognosis for better DFS and DMFS, and increment of CD4/CD8 ratio was a markedly powerful indicator of improved LRFS. Although FoxP3+ TIL was an independent prognostic factor for DMFS, we could not demonstrate any significance association between CD8+ TILs expression and clinical outcomes in MVA. In recent years, it has become clear that assessing immune infiltration is of greater prognostic significance than conventional Tumor, Node Metastasis (TNM) staging.[15] CD8+ cytotoxic T lymphocytes (CTLs) are directly capable of killing tumor cells and positively affect prognosis in a broad range of tumor types, including breast cancer, ovarian cancer, head and neck cancer and lung cancer et al [24-27]. In accordance with previous results, we demonstrated that higher CD8+ infiltration is associated with longer OS, DFS and DMFS in UVA. However, several other studies indicated that there is no such correlation with prognosis. One study even found a negative effect of CD8+ TIL on survival, but this did not reach statistical significance in multivariate analysis [28-30]. By contrast, as one of the paradoxically functional components of tumor-related immune system, FoxP3+ TIL is considered to be the most specific Treg marker that are involved in maintaining immune tolerance to the host. In tumor progression, Treg cells produce the inhibitory cytokines interleukin 10, transforming growth factor β and haemoglobin oxygenase 1 to achieve immune escape [31]. Therefore, many studies have suggested that higher FoxP3 Treg infiltration were associated with poor
prognosis in various malignancies including breast, lung, cervical, oral cavity and ovarian cancers [32, 33]. On the other hand, accumulating evidence has emerged that in others their presence was associated with better prognosis encompassing HPSCC [23, 34-36]. So far, the role of FoxP3 regulator T cells in cancer is still conflicting. Taken together, cytotoxic CD8+ T cells and regulatory FoxP3 T cells, as the two major components of the tumor-related immune system, merit obtaining more precise estimates of their effects on HPSCC patient survival. The present study also demonstrated that higher FoxP3 TIL density in UVA led to significantly better outcomes in OS, DFS and DMFS, but we have only explored an independent prognostic significance for DMFS in MVA, which is slightly different from our previous study [23]. Furthermore, the current data recapitulated the findings that CD4 TIL density had no impact on survival but showed strongly correlations with CD8 and FoxP3. We assumed that the presence of CD4 T-cells alone is not associated with the prognosis and may interact with other subsets exerting much more effects in the tumor microenvironment.

To measurement the relative number of TILs and the association between the different subsets, the data indicated that positive correlations were evidently among CD8, FoxP3 and CD4 T cells. As an indicator of the balance between CD8+ TILs and FoxP3 Treg cells in the tumor microenvironment, the ratio of CD8/FoxP3 appeared to be useful for predicting clinical outcomes. In reviewing of literatures, we conducted that CD8/FoxP3 ratio turned out to have a positive effect on prognosis in a number of tumors, including osteosarcoma, colorectal cancer and breast cancer [21, 33, 37-39]. For patients with tonsillar cancer, a high CD8/Foxp3+ ratio positively correlated with DFS [40]. Ni et al had reported that increased CD8/FoxP3 ratios were associated with improved OS, DFS and tumor stage in tongue cancer but was not an independent prognostic factor in MVA [28]. Similar to these studies, this cohort demonstrated that high CD8/FoxP3 ratio correlated with favorable prognosis and CD8 expression, which further confirmed that CD8+ TIL is associated with good prognosis in HPSCC patients. Also, when using different survival end points, the CD8/FoxP3 ratio consistently acted as an independent prognostic factor for DFS and DMFS in MVA. A large meta-analysis of TIL phenotyping, encompassing 33 studies and nearly 10,000 patients, indicated that lymphocyte ratios, particularly the CD8/FoxP3 ratio, have more prognostic potential than individual lymphocytic subtypes [31].
Although an investigation showed no significant correlation between CD8/FoxP3 ratio and survival in ovarian cancer [41], the ratio of CD8/FoxP3 was a promising prognostic marker in HPSCC. Additionally, the current study also suggested that high CD4/CD8 ratio was associated with better DMFS and LRFS in UVA and exhibited an independent prognostic factor for LRFS in MVA, albeit CD4 TIL density did not statistically correlate to survival implications. A study in lung cancer reached a similar conclusion, patients with higher CD4/CD8 ratios were associated with better OS, while CD4+ or CD8+ T cells counts separately did not significantly affected disease outcome [13]. Likewise, CD4/CD8 ratio can potentially be used for the same purpose in squamous cell carcinoma of the cervix [42]. However, there were also instances in which CD4/CD8 ratio was not linked to clinical outcomes, and some researchers even reported that high CD4/CD8 ratio was associated with tumor recurrence and the absence of lymphovascular invasion [28, 41, 43, 44]. The tendency for better clinical outcomes and progression in patients with high CD4/CD8 ratio is of note, despite the prognostic significance being limited to relatively few studies and insufficient for clinical use. It is also noteworthy that the current study found Foxp3 to be an independent prognostic factor for DMFS, whereas CD8 didn’t show any significance in MVA. These results were different from our previous study because lymphocyte ratios were included in MVA in this cohort, which supported the idea of the CD8/FoxP3 and CD4/CD8 ratios that produced a more impressive prognosis than each subtypes alone. Altogether, in the era of immunotherapy, these immune-biomarkers may provide new clues to therapeutic strategies and are speculated to be a possible predictive markers of treatment efficacy. Further studies are required to validate the results of the present study in a large cohort with neoadjuvant settings. Although the present results are very promising, previous data have shown that TILs are localized in all areas of tumor and have proven to be of prognostic value [15, 33]. The effects of TILs and the ratios between different subsets in stroma and the assessment of chemotherapeutic efficacy in patients with HPSCC still must still be evaluated.

Conclusion
This study demonstrated that high TILs are of prognostic significant in HPSCC, while the ratios
between these subsets may be more informative. We stressed that high ratio of CD8/Foxp3 accurately predict prognosis for improved DFS and DMFS, and increment of CD4/CD8 ratio was an independent prognostic factor for better LRFS. These findings will improve understanding the clinical significance of immune cells existing in HPSCC.

Abbreviations
AJCC: American Joint Committee on Cancer; CCRT: Concurrent chemoradiation therapy; CTLs: Cytotoxic T lymphocytes; DFS: Disease-free survival; DMFS: Distant metastasis-free survival; HE: Hematoxylin-eosin; HPSCC: Hypopharyngeal squamous cell carcinoma; IHC: Immunohistochemistry; LRFS: Local relapse-free survival; MVA: Multivariate analysis; NSCLC: Non-small cell lung cancer; OS: Overall survival; TNM: Tumor Node Metastasis; Tregs: Regulatory T cells; UVA: univariate analysis;

Declarations

Ethics approval and consent to participate
This study was approved by the Institutional Ethics Committee of The Science and Technology Commission of Shanghai Municipality, and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication
Not applicable.

Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
No author has financial or other contractual agreements that might cause conflicts of interest.

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Tables

Table 1 Clinicopathological factors of HPSCC with the status of CD8, CD4, FOXP3, the ratios of CD8/Foxp3 and CD4/CD8 (N = 132).
| Characteristics | N (%) | CD8 | | | | CD4 | | | | Foxp3 | | |
|----------------|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|                |       | low | high | P   | low | high | P   | low | high | P   | low | high |
| Age at diagnosis |     |     |     |     |     |     |     |     |     |     |     |     |
| <60y            | 71 (53.8) | 31  | 40  | 0.033* | 31  | 40  | 0.295 | 37  | 34  |
| ≥60y            | 61 (46.2) | 38  | 23  |       | 33  | 28  |       | 29  | 32  |
| Sex             |       |     |     |     |     |     |     |     |     |     |     |     |
| Male            | 131 (99.2) | 1   | 0   | 1.000 | 0   | 1   | 1.000 | 0   | 1   |
| Female          | 1 (0.8) | 68  | 63  |     | 64  | 67  |     | 66  | 65  |
| Smoke           |       |     |     |     |     |     |     |     |     |     |     |     |
| No              | 17 (12.9) | 12  | 5   | 0.124 | 5   | 12  | 0.120 | 7   | 10  |
| Yes             | 115 (87.1) | 57  | 58  |     | 59  | 56  |     | 59  | 56  |
| Drink           |       |     |     |     |     |     |     |     |     |     |     |     |
| No              | 28 (21.2) | 16  | 12  | 0.671 | 13  | 15  | 0.834 | 13  | 15  |
| Yes             | 104 (78.8) | 53  | 51  |     | 51  | 53  |     | 53  | 51  |
| Site            |       |     |     |     |     |     |     |     |     |     |     |     |
| Pyriform sinus  | 116 (87.9) | 57  | 59  | 0.064 | 54  | 62  | 0.290 | 54  | 62  |
| Not pyriform sinus | 16 (12.1) | 12  | 4   |     | 10  | 6   |     | 12  | 4   |
| Grade           |       |     |     |     |     |     |     |     |     |     |     |     |
| G1+G2           | 103 (78.0) | 61  | 42  | 0.003* | 55  | 48  | 0.037* | 59  | 44  |
| G3              | 29 (22.0) | 8   | 21  |     | 9   | 20  |     | 7   | 22  |
| Stage           |       |     |     |     |     |     |     |     |     |     |     |     |
| III             | 35 (26.5) | 15  | 20  | 0.238 | 15  | 20  | 0.554 | 12  | 23  |
| IVA/IVB         | 97 (73.5) | 54  | 43  |     | 49  | 48  |     | 54  | 43  |
| T stage         |       |     |     |     |     |     |     |     |     |     |     |     |
| T1-3            | 55 (41.7) | 40  | 44  | 0.205 | 39  | 45  | 0.589 | 36  | 48  |
| T4a             | 77 (58.3) | 29  | 19  |     | 25  | 23  |     | 30  | 18  |
| N stage         |       |     |     |     |     |     |     |     |     |     |     |     |
| N0-1            | 55 (41.7) | 29  | 26  | 1.000 | 25  | 30  | 0.599 | 24  | 31  |
| N2-3            | 77 (58.3) | 40  | 37  |     | 39  | 38  |     | 42  | 35  |
| Laryngectomy    |       |     |     |     |     |     |     |     |     |     |     |     |
| Total           | 77 (58.3) | 43  | 34  | 0.379 | 39  | 38  | 0.599 | 41  | 36  |
| Partial         | 55 (41.7) | 26  | 29  |     | 25  | 30  |     | 25  | 30  |
| CD8 (cut off: 80) |     |     |     |     |     |     |     |     |     |     |     |     |
| Low             | 69 (52.3) |     |     |     | 43  | 26  | 0.001* | 50  | 19  |
| High            | 63 (47.7) |     |     |     | 21  | 42  |     | 16  | 47  |
| CD4 (cut off: 30) |     |     |     |     |     |     |     |     |     |     |     |     |
| Low             | 64 (48.5) | 43  | 21  | 0.001* |     |     |     |     |     |
| High            | 68 (51.5) | 26  | 42  |     |     |     |     |     |     |
| Foxp3 (cut off: 30) |     |     |     |     |     |     |     |     |     |     |     |
| Low             | 66 (50.0) | 50  | 16 | <0.001* |     |     |     |     |     |
| High            | 66 (50.0) | 19  | 47  |     |     |     |     |     |     |

Abbreviations: HPSCC, hypopharyngeal squamous cell carcinoma; G1, well differentiated; G2, moderately differentiated, G3, poorly differentiated.

*The P value is significant

**Table 2 Univariate analyses of OS, DFS, DMFS and LRFS of the entire population (N = 132).**
| Variables                      | OS      | DFS      |
|--------------------------------|---------|----------|
|                                | HR      | 95%CI    | P     | HR      | 95%CI    | P     |
| Age, years (≥60 y vs <60 y)    | 0.521   | 0.254-1.073  | 0.077 | 0.637   | 0.341-1.188  | 0.15  |
| Smoke (Yes vs No)              | 0.596   | 0.259-1.372  | 0.224 | 0.738   | 0.328-1.662  | 0.46  |
| Drink history (Yes vs No)      | 0.748   | 0.348-1.607  | 0.457 | 0.945   | 0.452-1.976  | 0.88  |
| Site (Not PS vs PS)            | 2.534   | 1.144-5.610  | 0.022*| 2.524   | 1.206-5.284  | 0.01  |
| Grade (G3 vs G2+G1)            | 0.689   | 0.739-1.665  | 0.408 | 1.033   | 1.035-2.102  | 0.92  |
| Stage (IVA/IVB vs III)         | 1.785   | 4.313-1.151  | 0.198 | 2.457   | 5.834-1.365  | 0.04  |
| T stage (T4a vs T1-3)          | 2.259   | 4.436-0.652  | 0.018*| 2.508   | 4.610-0.874  | 0.00  |
| N stage (N2-3 vs N0-1)         | 1.319   | 2.666-0.853  | 0.441 | 1.682   | 3.236-0.951  | 0.12  |
| Laryngectomy (Total vs Partial)| 1.896   | 4.215-1.127  | 0.117 | 1.860   | 3.638-1.005  | 0.07  |
| CD8 (Low vs High)              | 2.324   | 4.795-0.593  | 0.022*| 1.892   | 3.560-0.610  | 0.04  |
| CD4 (Low vs High)              | 1.174   | 2.323-1.511  | 0.645 | 1.119   | 2.052-1.063  | 0.71  |
| Foxp3 (Low vs High)            | 3.253   | 7.001-0.752  | 0.003*| 1.999   | 3.760-1.159  | 0.03  |
| CD8/Foxp3 (Low vs High)        | 1.490   | 2.953-0.270  | 0.253 | 2.205   | 4.195-0.260  | 0.01  |
| CD4/CD8 (Low vs High)          | 0.539   | 1.075-0.853  | 0.079 | 0.486   | 0.910-0.107  | 0.02  |

Abbreviations: PS, pyriform sinus; OS, overall survival; DFS, disease-free survival; DMFS, distant metastasis-free survival; LRFS, local relapse-free survival; G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

*The P value is significant

**Tabel 3 Multivariate analyses of OS, DFS, DMFS and LRFS of the entire population (N = 132).**

| Variables                      | OS      | DFS      |
|--------------------------------|---------|----------|
|                                | HR      | 95%CI    | P     | HR      | 95%CI    | P     |
| Site (Not PS vs PS)            | 1.621   | 0.706-3.726  | 0.255 | 1.756   | 0.810-3.807  | 0.154 |
| Stage (IVA/IVB vs III)         | 1.880   | 0.947-3.735  | 0.071 | 2.196   | 1.077-4.476  | 0.030*|
| T stage (T4a vs T1-3)          | 1.231   | 0.509-2.977  | 0.644 | 0.716   | 0.300-1.709  | 0.452 |
| CD8 (Low vs High)              | 2.387   | 0.932-6.111  | 0.070 | 2.066   | 0.885-4.826  | 0.094 |
| Foxp3 (Low vs High)            | 2.613   | 1.203-5.673  | 0.015*| 2.613   | 1.203-5.673  | 0.015*|
| CD8/Foxp3 (Low vs High)        | 0.586   | 0.299-1.148  | 0.119 | 0.586   | 0.299-1.148  | 0.119 |

Multivariate cox regression analyses were performed for all variables that were significantly associated with survival in univariate analysis.
Abbreviations: PS, pyriform sinus; OS, overall survival; DFS, disease-free survival; DMFS, distant metastasis-free survival; LRFS, local relapse-free survival; G1, well differentiated; G2, moderately differentiated, G3, poorly differentiated.

*The P value is significant

Figures
Representative images for immunohistochemical detection of tumor-infiltrating T lymphocytes are shown (Fig. 1A-F).
We also found markedly correlations among CD8, CD4 and FoxP3, using Pearson’s correlation coefficient (P<0.001, Fig. 2A-C).
Significant differences were found between the high and low CD8+TIL groups in 3-year OS, DFS and DMFS, but not in the LRFS (Fig. 3A-D). Similarly, a higher FOXP3+TIL was also strongly correlated with better OS, DFS and DMFS (P=0.001, P= 0.028 and P=0.009, respectively, Fig. 3E-H).
Further analysis revealed that patients with a high CD8/FoxP3 ratio had significantly better DFS and DMFS (P=0.013 and P= 0.029, respectively) (Fig. 4B-C), while higher ratio of CD4/CD8 had evidently improved 3-year DFS and LRFS compared with lower CD4/CD8 ratio (P = 0.029 and P = 0.033, respectively) (Fig. 4F, H). By contrast, no association between the status of CD8/FoxP3 or CD4/CD8 ratio and OS was observed (Fig. 4A, E).