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Analysis of Th1/Th2 cytokine and clinical characteristics with head and neck squamous cell carcinoma

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ABSTRACT

A retrospective analysis was conducted on 58 patients with laryngeal and hypopharyngeal tumors and 27 patients with benign vocal cord lesions to explore the role of serum cytokines in these diseases' characteristics and immunotherapy. The differences in the levels of 12 cytokines were measured. Additionally, the study examined the correlation between T helper cells (Th)1/Th2 cytokine levels and the clinical characteristics and immunotherapy efficacy of laryngeal and hypopharyngeal cancers. The results show that the balance of Th1/Th2 is biased towards Th2 in patients with laryngeal and hypopharyngeal tumors. Among these, Interleukin (IL)-6 (P = 0.021) is highly expressed in laryngeal tumors, and the expression levels of IL-1β (P = 0.008), IL-6 (P = 0.005), and IL-8 (P = 0.05) are higher in patients with poorly differentiated laryngeal tumors. The level of IL-4 (P = 0.0048) is significantly correlated with tumor location. The expression levels of IL-2 (P = 0.010), IL-4 (P = 0.028), IL-10 (P = 0.011), IL-12p70 (P = 0.034), IL-17 (P = 0.024), Tumor Necrosis Factor (TNF)-α (P = 0.003), and Interferon (IFN)-γ (P = 0.007) are related to lymph node metastasis. The level of IFN-γ (P = 0.016) is correlated with the efficacy of neoadjuvant therapy, while the level of IFN-α (P = 0.013) is significantly correlated with Programmed Death Ligand 1 (PD-L1) expression. The

Principal Component Analysis (PCA) results showed that patients with tumors, poor differentiation, and lymph node metastasis had higher levels of Th1 and Th2 cytokine separation. In conclusion, the shift in the balance of Th1 and Th2 cytokine expression indicates higher tumor invasiveness, and IFN has potential as a circulating molecular marker for immunotherapy of head and neck squamous cell carcinoma.

KEYWORDS： Cytokine, Th1/Th2 cytokine, head and neck squamous cell carcinoma, IL-1β, Interferon.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) has a high incidence rate and is the sixth most common cancer in the world. Due to the insidious and high invasiveness of the disease, about 60% of patients are diagnosed in the late stage of the disease, and the overall prognosis is poor[1]. Research on tumor immunity has made significant progress in multiple tumor fields, and immunotherapy has also been developed on this basis. However, only a small number of patients benefit from this therapy of advanced pharyngeal squamous cell carcinoma. For example, only about 20% to 30% of patients truly benefit from immune checkpoint inhibitors

(ICIs) alone. Therefore, more comprehensive and accurate research is still needed on the tumor immune status of patients with HNSCC.

Cytokines (CK) promote or inhibit malignant tumors by binding to different receptors and activating corresponding signaling pathways. They also play a regulatory role in various tumor immune microenvironments and are one of the potential biomarkers for disease risk prediction. This article analyzes the correlation between serum cytokine levels and clinical characteristics in patients with laryngopharynx squamous cell carcinoma, and explores the role of cytokines in HNSCC.

MATERIALS AND METHODS

Material

Collect patients admitted to the Department of Otolaryngology, Head and Neck Surgery at Beijing Tongren Hospital affiliated with Capital Medical University from January 1, 2023 to August 1, 2023, including 58 patients with laryngopharynx squamous cell carcinoma and 27 patients with benign vocal cord lesions (vocal cord leukoplakia/polyps). Serological cytokine tests were performed on 85 patients, including serum IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17, IL-12p70, TNF-α, IFN-α, IFN-γ. Divide 12 cytokines into TH1 cytokines: IL-2, IL-

12p70, IFN-γ, IFN-α, TNF-α; And TH2 cytokines: IL-4, IL-6, IL-5, IL-8, IL-10. Collect general information of patients, including clinical characteristics of tumor patients: age, smoking history, drinking history, tumor location, differentiation degree, AJCC stage, lymph tissue metastasis, and treatment methods. All methods were carried out in accordance with relevant guidelines and regulations. All patients provided written informed consent and for minors, parents or legal authorized representative provided written informed consent. The study was approved by the Ethics Committee of Beijing Tongren Hospital (TRECKY2021-050).

Cytokine detection

The 12-in-1 cytokine detection kit from Qingdao Resker Biotechnology Co., Ltd. was used to evaluate serum samples from patients using a multiplex microsphere flow immunofluorescence assay. All operations are strictly carried out in accordance with the product manual. Samples were prepared by combining 25μL of serum with 75μL of sample solution (comprising 25μL each of measuring buffer, capture microsphere antibodies, and test antibodies for IL-1β, IL-6, IL-12, IL-17, IL-8, IL-5, IL-2, IL-4, IL-10, TNF-α, IFN-α, IFN-γ). This mixture was incubated at room temperature (25 ± 1 ℃) in the dark, without vibration, for 2 hours (approximately 400-

500rpm). After adding 25μL SA-PE, incubation continued under the same conditions for 30 minutes. Tubes were then washed with 1000μL buffer, centrifuged at 300-500xg for 5 minutes, and the supernatant was discarded. Tubes were inverted to dry on absorbent paper. The microspheres were resuspended in 150-300μL wash buffer by vortexing for 10 seconds, and samples were analyzed immediately using flow cytometry.

Neoadjuvant therapy

The patient received neoadjuvant therapy: PD-1 inhibitor combined with chemotherapy for 1-2 cycles. On the first day of each cycle, PD-1 inhibitor pembrolizumab (200 mg) is intravenously injected. The chemotherapy regimen includes paclitaxel and nedaplatin. Starting from day 2, paclitaxel 210mg is administered intravenously, and nedaplatin 40mg is administered intravenously from day 3-5. According to the internationally recognized criteria for evaluating the efficacy of immunotherapy in solid tumors (RECIST 1.1), patients are evaluated for complete response (CR) through imaging examinations. For solid tumors other than nodular diseases, all target lesions (target lesions) disappear completely, or all target nodules (target nodules) must shrink to normal size (short axis<10 mm) and last for 4 weeks or more. Partial Response (PR): The sum of the diameters of all measurable target lesions (long

diameter for the target lesion and short diameter for the target nodule) decreases by ≥ 30% compared to baseline and lasts for 4 weeks or more. Progressive disease (PD): The minimum value of the sum of all measured target lesion diameters throughout the entire tumor treatment process is used as the reference value. Patients whose sum of target lesion diameters exceeds the reference value by 20% or more, and the absolute value increases by 5 mm or more, or if one or more new lesions appear and complete or partial remission is not achieved before the volume or quantity of lesions increases, are considered as disease progression. Stable disease (SD): Changes in lesion volume and quantity between partial remission and disease progression can be considered as disease stability.

Immunohistochemistry

For PD-L1 antibody detection and CPS scoring, tissues were fixed in 10% neutral buffered formalin at room temperature (15-25 ℃) for 6-72 hours. The VENTANA PD-L1 (SP263) Assay was employed using the BenchMark ULTRA system. Tissues were processed by dewaxing at 60 °C for 12 minutes, followed by antigen retrieval with Cell Conditioning Solution (CC1) for 64 minutes. VENTANA PD-L1 (SP263) was used as the primary antibody, incubated at 36 °C for 16 minutes. This was followed by an 8-minute incubation with OptiView

HQ Linker and Multimer. Staining was completed with Hematoxylin II for 4-8 minutes and a blue reagent for 4 minutes to achieve the final immunohistochemical visualization.

For P53, P16, P63, and Ki-67 detection using the PV-6000 kit, paraffin-embedded sections were dewaxed and rehydrated. Endogenous peroxidase activity was quenched by incubation in 3% H2O2 for 5-10 minutes at room temperature. Sections were washed and soaked in PBS for 5 minutes, followed by antigen retrieval in a pressure cooker for 2.5-3 minutes. Primary antibodies were applied and incubated at 37 ℃ for 1-2 hours or overnight at 4 ℃. After washing, secondary antibody from the PV-6000 kit was added and incubated for 30 minutes at room temperature. Visualization was achieved with DAB and sections were then washed, counterstained, dehydrated, cleared, and mounted.

UALCAN analysis

UALCAN is a comprehensive and interactive web resource that provides easy access to publicly available cancer omics data (http://ualcan.path.uab.edu/index.html). Here, the mRNA and expression for genes IL-1β, IL-6, IL-12, IL-17, IL-8, IL-5, IL-2, IL-4, IL-10, TNF-α, IFN-α, and IFN-γ in head and neck squamous cell carcinoma was evaluated using TCGA databases.

Statistical analysis

Statistical analysis was performed using SPSS version 24.0 and R version 4.0.2 (available at https://www.R-project.org). Variables with a normal distribution were analyzed using independent sample t-tests, while categorical variables were examined using the chi-square test. Correlations and effects between various cytokine levels and clinical features were estimated. All p-values were two-tailed, with $p < 0.05$ considered statistically significant. Principal Component Analysis (PCA) was conducted using the "limma" and "scatterplot3d" packages in R for analysis and visualization.

RESULTS

General information

A total of 85 patients were included in the study (Table 1), including 58 patients with pharyngeal cancer and 27 patients with benign vocal cord lesions. Among the patients with laryngopharynx squamous cell carcinoma, there were 33 cases of laryngeal cancer and 25 cases of hypopharyngeal cancer, with a median age of 63.5 years. Among them, 11 patients received preoperative anti PD-L1 therapy combined with chemotherapy, and 47 patients received direct

surgical treatment. 27 patients with benign vocal cord lesions, including vocal cord keratosis, vocal cord polyps, and vocal cord dysplasia.

Correlation analysis between serum cytokine levels and different diseases

Comparing the serum cytokine levels of 58 patients with pharyngeal cancer and 27 patients with benign vocal cord lesions, the results showed that the expression levels of IL-1 β, IL-6, IL-8, IFN-γ IL-2, IL-4, IL-5, IL-10, IL-17, IL-12p70, IFN-α, and TNF-α are higher in tumor patients than in benign patients. Among them, there was a statistically significant difference in the expression of IL-4 (p=0.0130), IL-6 (p=0.0213), IL-10 (p=0.0399), IL-12p70 (p=0.0466), and TNF-α (p=0.0297) (Table 2). IL-6 is highly expressed in head and neck squamous cell carcinoma, while other cytokines are highly expressed in benign lesions (Figure 1A). The balance drift of Th1/Th2 in benign patients leans towards Th1, while in tumor patients, the balance drift of Th1/Th2 leans towards Th2. Through the principal component analysis (PCA), it can be seen that there is no significant separation of Th1 and Th2 cytokines in the benign group, while in patients with head and neck squamous cell carcinoma, there is a significant difference in Th1 and Th2 cytokines on the PC2 axis (Figure 2. AB), indicating a significant

Th1/Th2 balance drift in the systemic inflammatory state of tumor patients. Overall, immunosuppressive cytokines are highly expressed in tumor patients.

Using UALCAN to analyze the expression of various cytokines in head and neck squamous cell carcinoma in the TCGA database, it can be found that IL-1β, IL-10 and TNF are highly expressed in head and neck tumor tissues, with statistically significant differences ($p<0.01$); The expression differences of IL-2, IL-6, and IL-8 in head and neck tumors and normal tissues were not statistically significant (Figure 3). Combined with the results of this study, it can be found that there are differences in the protein levels of serum cytokines and mRNA expression levels in tissues, among which the expression of IL-1β presents consistency.

Correlation analysis between serum cytokine levels and clinical characteristics of tumors

The correlation between serum cytokine levels and tumor differentiation degree

Among 58 patients with laryngeal squamous cell carcinoma, 29 (50%) were poorly differentiated. The correlation analysis between the levels of 12 cytokines in the patient's serum and the degree of tumor differentiation showed that the levels of IL-1β ($p=0.008$), IL-6 ($p=0.005$), and IL-8 ($p=0.05$) were significantly correlated with the degree of tumor differentiation (Figure 1B) (Table 3), and the balance drift of Th1/Th2 in poorly differentiated

tumors was biased towards Th2. Through the principal component analysis (PCA), it can be seen that compared to the medium to high differentiation group, the expression levels of the two types of cytokines in the low differentiation group have a more significant difference on the PC2 axis (Figure 2. CD). In poorly differentiated tumors, there is a more significant immunosuppressive state, and related cytokines are highly expressed.

Analysis of differences in serum cytokine levels between laryngeal cancer and hypopharyngeal cancer

Among 58 patients with squamous cell carcinoma of the throat, there were 33 patients with laryngeal cancer and 25 patients with hypopharyngeal cancer. A correlation analysis was conducted between the levels of 12 cytokines and tumor location. The results showed (Table 3) that IL-4 ($p=0.0048$) levels were significantly correlated with tumor location (Figure 1. D). The expression level of IL-4 was higher in hypopharyngeal carcinoma than in laryngeal carcinoma, and this immunosuppressive state may be related to a poorer prognosis in nasopharyngeal carcinoma.

Correlation between serum cytokine levels and tumor lymph node metastasis

Among 58 patients with 25 patients (43.10%) had lymph node metastasis. Correlation analysis was conducted between the levels of 12 cytokines and lymph node metastasis. The results showed that the level of IL-2 ($p=0.025$), IL-4 ($p=0.005$), IL-8 ($p=0.009$), IL-10 ($p=0.017$), IL-12p70 ($p=0.002$), IL-17 ($p<0.001$), TNF-α ($p<0.001$), and IFN-γ ($p=0.026$) is significantly correlated with lymph node metastasis (Figure 1. C). Multiple logistic regression was performed to compare the seven cytokines mentioned above with factors that may affect lymph node metastasis, such as age, gender, smoking history, drinking history, location, T stage, differentiation, etc. The results showed that there is a statistically significant correlation between the expression level of IL-2 ($p=0.010$), IL-4 ($p=0.028$), IL-10 ($p=0.011$), IL-12p70 ($p=0.034$), IL-17 ($p=0.024$), TNF-α ($p=0.003$), and IFN-γ ($p=0.007$) and lymph node metastasis (Table 3). The PCA results showed that compared to the lymph node metastasis negative group, the expression levels of Th1 and Th2 cytokines in the lymph node metastasis positive group showed significant differences on the PC2 axis (Figure2 E-F). The overall expression of cytokines in patients with lymph node metastasis is at a high level, which is related to tumor progression, mobilization of cellular and humoral immunity in the body.

There was no statistically significant correlation analysis between the levels of 12 cytokines in the patient's serum and tumor staging.

Serum cytokines and neoadjuvant therapy

Among 58 patients, 11 patients underwent neoadjuvant therapy with pembrolizumab combined with chemotherapy, of which 5 underwent 2 cycles of treatment and 6 underwent 1 cycle of treatment. Partial Response (PR) in 7 patients and Stable Disease (SD) in 4 patients. Analyze the levels of 12 cytokines in the serum before and after receiving immunotherapy. The results showed that the expression levels of IL-2 ($p=0.016$) and IFN-γ ($p=0.016$), IL-4 ($p=0.016$) and IL-10 ($p=0.031$) in PR group were significantly increased before treatment (Table 4 and Figure 4), while there was no significant difference in the 12 cytokines before and after immunotherapy in the SD group patients.

The correlation between serum cytokine levels and tumor markers

A correlation analysis was conducted between the levels of 12 cytokines and tumor markers in 58 patients. The results showed that IL-2 ($p=0.002$), IL-6 ($p=0.036$), IL-10 ($p=0.022$), IL-12p70 ($p=0.042$), and IFN-α ($p=0.001$) were significantly negatively correlated with the positive expression of p16 in tumor tissue; IL-2 ($p=0.039$) was significantly negatively

correlated with positive expression of p63 in tumor tissue; IL-8 (p=0.028) is significantly positively correlated with the expression of Ki-67 in tumor tissue; The level of IFN-α (p=0.013) is significantly positively correlated with the CPS index of PD-L1in peripheral blood. The correlation between PD-L1 expression and its level indicates its potential to participate in anti-tumor immunity (Table 5).

DISCUSSION

Cytokines can be classified as pro-inflammatory or anti-inflammatory based on their effects on inflammation[2], the former including IL-1β、IL-6 and TNF- α etc; The latter includes IL-4, IL-10, IL-17, etc. T lymphocytes are the main source of cytokines, and CD4+T lymphocytes, also known as helper T cells (Th cells), are considered as cytokine producers. Mossman et al. [3] described CD4+Th cell subpopulations and named them TH1, TH2, TH9, TH17, TH22, or follicular TH cells based on the different cytokines they produce. These T cell subsets can promote different types of inflammatory responses based on their respective cytokine profiles, responses to chemokines, and interactions with other cells. Th1 cytokines mainly include interferon-γ (IFN)-γ、Tumor Necrosis Factor (TNF)-α、TNF-β、Cytokines such as interleukin-2 (IL-2) and interleukin-12 (IL-12); These cytokines can promote T cell-mediated immune

response, which is cellular immunity, releasing cytokines that cause inflammation and mediating cellular immunity. Th2 cytokines mainly include cytokines such as interleukin-4 (IL-4), interleukin-6 (IL-6), and interleukin-10 (IL-10); The main function of these cytokines is to mediate the humoral immune response and inhibit the Th1 response. When the body is in a normal state, the number of Th1 and Th2 cells is in a dynamic equilibrium state. Th1 and Th2 secrete cytokines, cross regulate and inhibit each other, maintain normal cellular and humoral immune functions, and maintain a balance in the human immune system. When the body experiences functional abnormalities, Th1 and Th2 often exhibit a balance bias towards one side, known as "Th1/Th2 drift". The balance state is disrupted, causing the dynamic balance of the human cytokine network to be disrupted. In the body of tumor patients, there is a balance drift of Th1/Th2. When Th2 cytokines dominate, tumor immune escape occurs.

Research has shown that changes in cytokine expression play an important role in the malignant transformation of many cancers, including HNSCC[2]. Due to the excessive production of some cytokines by tumor cells, they can serve as important diagnostic markers in the serum of HNSCC patients, thus their research value is gradually highlighted. Evidence suggests that the development of HNSCC is associated with a decrease in Th1 cytokine levels and an increase

in Th2 cytokine levels, which is a mechanism for evading anti-tumor immune responses and affecting tumor growth [4]. This shift towards Th2 cytokine response is a common occurrence in many other solid tumors, such as colorectal cancer, renal cell carcinoma, prostate cancer, and melanoma. In this study, the expression levels of Th1 cytokines IL-12p70 and TNF-α were significantly higher in benign lesions than in patients with pharyngeal squamous cell carcinoma. The expression levels of Th2 cytokines IL-6 and IL-8 were higher in pharyngeal squamous cell carcinoma than in benign lesions, which is consistent with previous literature reports [5].

In this study, we also found that IL-4 and IL-10, as members of the TH2 class of cytokines, were more expressed in the benign lesion group. At the same time, in immunotherapy patients who responded well, IL-4 and IL-10 significantly increased after treatment. This is contrary to the previously agreed conclusion. However, there are currently some cancer-related studies, including head and neck squamous cell carcinoma, that have demonstrated the biological functions of these two cytokines that differ from other TH2 cytokines. Hoffmann et al.[6] found that compared to the 20 control group, the serum levels of IL-4 and IL-10 did not significantly increase in 20 patients with head and neck squamous cell carcinoma. In a study of 93 patients with head and neck squamous cell carcinoma and 53 healthy controls, no significant increase

in IL-10 was observed in the malignant patient group [7]. Some literature mentions that the anti-tumor effect of IL-4 contradicts previous evidence of promoting tumors, possibly due to the level of IL-4 and its interrelationships with other immune regulatory factors[8]. A study on advanced head and neck squamous cell carcinoma showed that the expression level of IL-4 were closely correlated with IL-2 and IFN-γ[9]. Some scholars also believe that it is likely due to the specific dynamics (half-life), metabolism, and orbital protein regulatory parameters of each individual HNSCC tumor[10].

The high secretion level of cytokines also participates in the indirect regulation of immune response and tumor cell differentiation process[11]. Among them, dysregulation of IL-6 signaling usually plays a core role in the differentiation of head and neck squamous cell carcinoma tumor cells [12]. Meanwhile, IL-1β also plays an important role in the differentiation of laryngeal squamous cell carcinoma. We know that one of the biological effects of IL-1 is to trigger an increase in the expression of IL-6, etc. Previous studies on the occurrence and development of HNSCC have clearly shown that serum IL-1 in patients β The increase in concentration is positively correlated with tumor staging, and its stimulation promotes cell proliferation, colony formation, and tumorigenicity. It can promote the stem cell characteristics

of HNSCC cells by activating the Smad/ID1 signaling pathway[13]. In this study, it was also demonstrated that IL-1 β、 IL-6 and IL-8 are abnormally overexpressed in poorly differentiated laryngeal squamous cell carcinoma, and there is a significant difference compared to the control group.

Among pro-inflammatory cytokines, IL-8 is a part of the CXC chemokine family and has been reported to play an important role in cancer invasion, angiogenesis, and metastasis[14,15]. In some cancers, such as breast cancer, colon cancer, cervical cancer, pancreatic cancer and leukemia, it has been proved that IL-8 can be secreted by cancer cells themselves or paracrine. In the past decade, four studies have focused on the role of IL-8 in LSCC, recruiting a total of 220 LSCC patients[16-19]. These studies indicate that the elevated levels of IL-8 cytokines in cancer patients are mainly related to tumor size and lymph node metastasis[17]. Recent studies have shown that lymph node metastasis can accelerate the hematogenous metastasis of tumors by affecting systemic immune surveillance[20]. High expression of MHC-II was observed in human lymph node metastasis and correlated with IFN-γ Related, some scholars believe that IFN induced by tumor cells entering lymph nodes- γ Signal transduction upregulates MHC-II, leading to local immune suppression formed by Treg infiltration to enhance LN seeding[21].

Researchers selected malignant cells from squamous cell carcinoma of the head and neck, and compared the transcriptome of lymph node metastasis and non lymph node metastasis. They found significant changes in IFN related genes[20]. In this study, IFN was analyzed through single factor and multiple factor analysis- γ The expression is significantly elevated in patients with head and neck squamous cell carcinoma with lymph node metastasis. Further confirms the IFN-γ Causing local immune suppression in lymph node metastasis, there is immune editing of lymph nodes after tumor cell invasion.

In head and neck squamous cell carcinoma, D M. Lathers et al. reported for the first time that differences in cytokine changes are related to the subpopulations of primary tumors[9]. IL-10 expression is highest in nasopharyngeal carcinoma, followed by laryngeal carcinoma. However, Nadine M. et al. believe that the expression of specific serum cytokines in HNSCC patients is consistent regardless of the tumor site [22]. This study demonstrates that compared to laryngeal cancer, IL-4, as a classic Th2 class cytokine, is highly expressed and has significant differences in hypopharyngeal cancer. The high expression of its immunosuppressive cytokines may be one of the reasons for the poorer prognosis of hypopharyngeal cancer compared to laryngeal cancer.

Serum cytokines as molecular markers have been highly valued by researchers in evaluating the efficacy of tumor immunotherapy[1]. A research team retrospectively analyzed 1344 phase III clinical trials of advanced cancer treated with anti-PD-1 antibodies and anti-CTLA-4 antibodies. The results showed that high levels of serum IL-8 expression were associated with poor cancer prognosis and reduced clinical efficacy[23]. Andresa S Laino et al. analyzed the serum of patients from three large phase II/III randomized ICI trials and found that higher baseline IL-6 levels were associated with shorter survival[24]. There are also studies showing that IFN is found in patients with tumor remission after treatment with anti-PD-1 monoclonal antibodies- γ The increase in expression indicates a correlation with better immunotherapy efficacy[8].

This study detected the expression of PD-L1 in tumor tissue of patients with pharyngeal squamous cell carcinoma. The Combined Positive Score (CPS) was used, and its correlation with cytokines in the patient's serum was analyzed. The results showed that the expression of IFN-α and PD-L1 in the patient's serum was significantly correlated, and IFN-γ Related to the efficacy of neoadjuvant therapy. Previous reports have shown that type I interferon IFN- α Participated in the regulation of PD-L1 by activating the PI3K-AKT mTOR signaling pathway

to participate in interferon dependent mRNA transcription, however, relevant reports are scarce in laryngeal squamous cell carcinoma. This article is the first to report a significant correlation between IFN-α and PD-L1 expression. IFN-γ/ The JAKs/STATs/IRF1 axis is the main regulatory mode of PD-L1 expression, and type II interferon IFN-γ It is the main inducer of PD-L1, IFN-γ The JAK/STAT1/IRF1 pathway plays a role in various types of cancer, such as melanoma, non-small cell lung cancer, liver cancer, head and neck squamous cell carcinoma, and gastric cancer [25,26]. The serum IFN level may be a potential biomarker for identifying the progression of anti-PD-1 monoclonal antibody therapy in head and neck squamous cell carcinoma. However, we still need more immunotherapy samples to confirm this finding.

CONCLUSION

The conclusion of this study indicates that patients with pharyngeal tumors have a Th1/Th2 balance drift biased towards Th2, and the degree of separation between Th1 and Th2 cytokines is higher in patients with head and neck squamous cell carcinoma and higher malignancy. In the future, the goal of further research is to use the ratio between the two types of cytokines as an effective indicator for clinical and pathological evaluation of cancer patients. In addition,

IFN has the potential to serve as a valuable circulating molecular marker for the efficacy of immunotherapy in head and neck squamous cell carcinoma.

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Ethics approval and consent to participate

All patients provided written informed consent and for minors, parents or legal authorized representative provided written informed consent. The study was approved by the Ethics Committee of Beijing Tongren Hospital (TREC2023-KY009).

Consent for publication

All the authors have consent for publication.

Authors' contributions

Wang Rong and Yin Gaofei: writing – original draft; Guo Wei: writing – original draft and supervision; Li Nuan and Wang Rong: data curation, formal analysis; Chen Xiaohong and Zhang Yang：supervision；Huang Zhigang：funding acquisition and writing – review & editing.

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TABLES AND FIGURES WITH LEGENDS

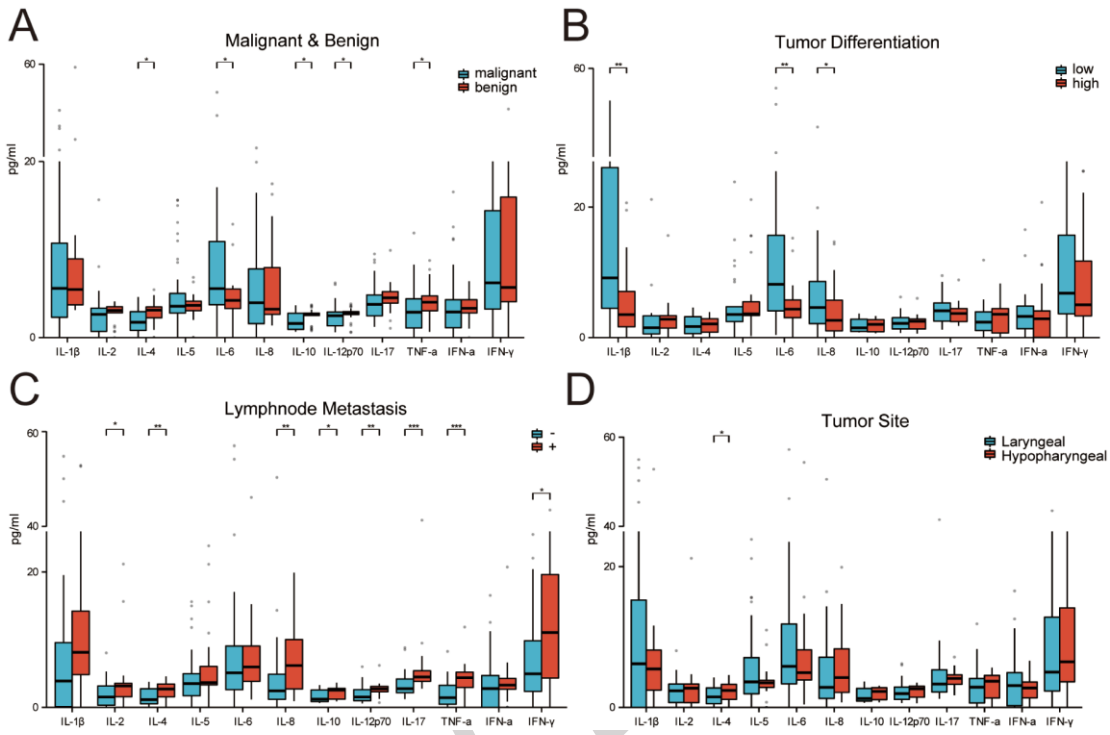

Figure 1. (A) There is a correlation between cytokines and tumor malignancy, including IL-4 (p=0.0130), IL-6 (p=0.0213), IL-10 (p=0.0399), IL-12p70 (p=0.0466), and TNF-α (p=0.0297); (B) Correlation between cytokines and tumor differentiation, IL-1 β (p=0.008), IL-6 (p=0.005), and IL-8 (p=0.05); (C) Correlation between cytokines and lymph node metastasis, IL-2 (p=0.010), IL-4 (p=0.028), IL-10 (p=0.011), IL-12p70 (p=0.034), IL-17 (p=0.024), TNF-α (p=0.003), IFN-γ (p=0.007). (D) Correlation between cytokines and tumor type, IL-4 (p=0.0048).

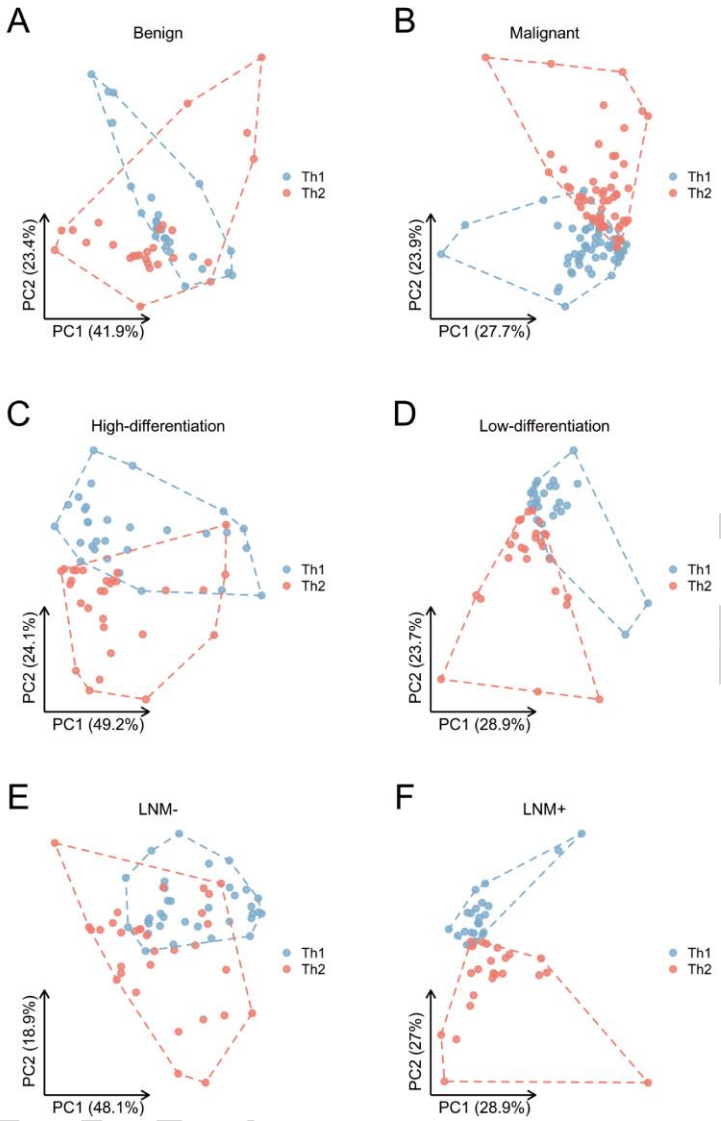

Figure 2. Grouping analysis of serum 12 cytokines TH1 and TH2 in patients with benign and malignant lesions showed that the malignant lesion group had a higher degree of separation between the two cytokines. Meanwhile, in the head and neck squamous cell carcinoma group, the higher the degree of malignancy (low differentiation and positive lymph node metastasis), the higher the degree of separation between the two types of cytokines. (A) Principal component analysis of TH1 and TH2 cytokines in the benign lesion group; (B) Principal

component analysis of TH1 and TH2 cytokines in HNSCC group; (C) Principal component analysis of TH1 and TH2 cytokines in the medium to high differentiation group; (D) Principal component analysis of TH1 and TH2 cytokines in the poorly differentiated group; E: Principal component analysis of TH1 and TH2 cytokines in the negative lymph node metastasis group; F: Principal component analysis of TH1 and TH2 cytokines in lymph node metastasis positive group.

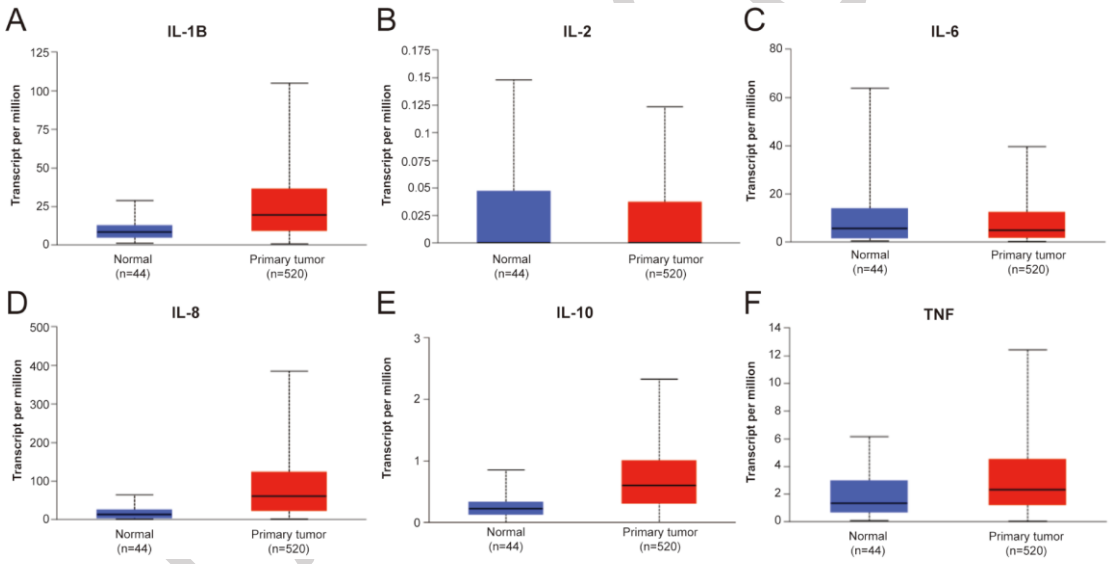

Figure 3. (A) IL-1β the difference in expression between head and neck tumors and normal tissues is statistically significant, $p<0.01$; (B) The expression difference of IL-2 in head and neck tumors and normal tissues is not statistically significant, $p>0.05$; (C) The expression difference of IL-6 in head and neck tumors and normal tissues is not statistically significant,

p>0.05; (D) The expression difference of IL-8 in head and neck tumors and normal tissues is not statistically significant, p>0.05; (E) The expression difference of IL-10 in head and neck tumors and normal tissues is statistically significant, p<0.01; (F) The expression difference of TNF in head and neck tumors and normal tissues is statistically significant, p<0.01.

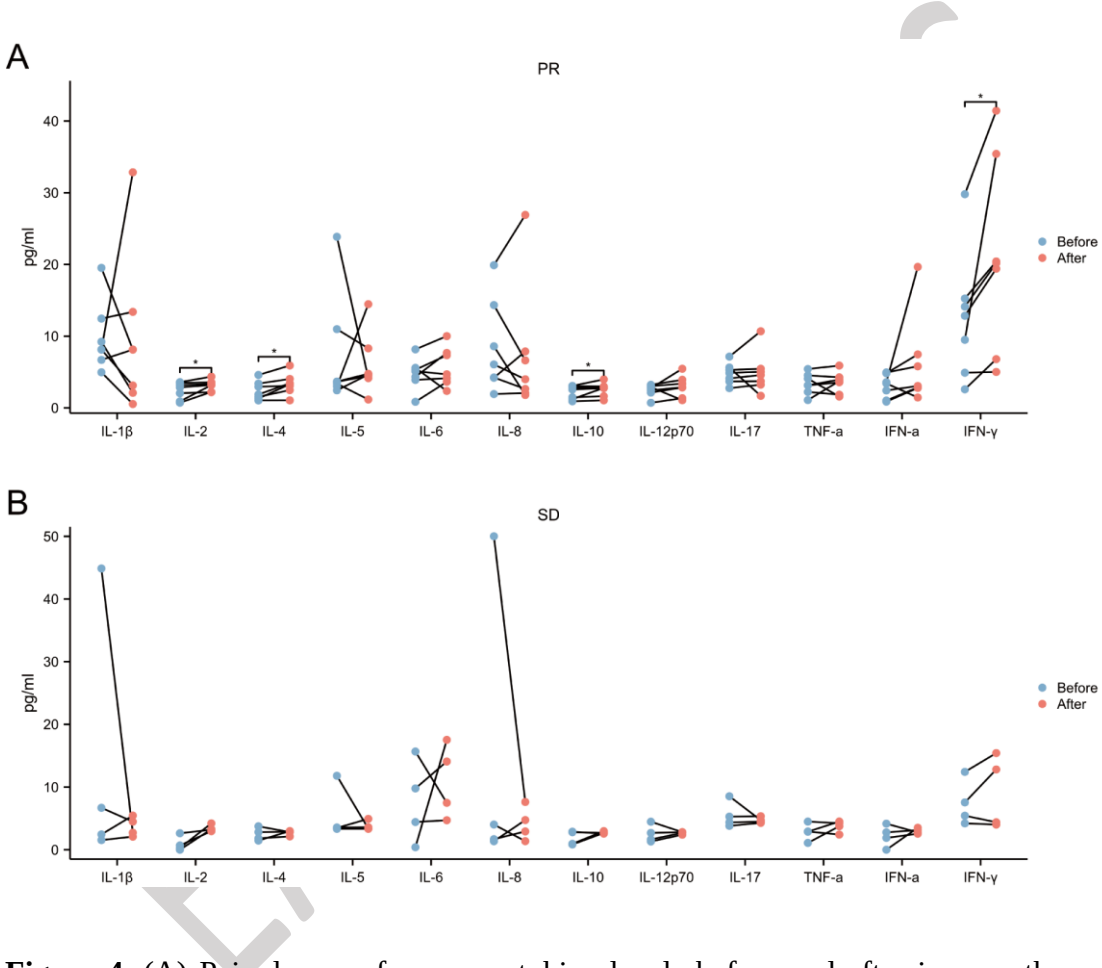

Figure 4. (A) Paired map of serum cytokine levels before and after immunotherapy in PR group patients; (B) Paired map of serum cytokine levels before and after immunotherapy in SD group patients. IL-2 (p=0.016), IFN-γ (p=0.016), IL-4 (p=0.016), IL-10 (p=0.031)

Table 1. General information

Clinical Feature	Grouping	Tumor（58）	Benign（27）
Age	≤45	2	12
	(45-60]	22	10
	(60-75]	28	0
	＞75	6	5
Sex	Female	1	3
	Male	57	24
Smoking	Yes	49	11
	No	9	16
Drinking	Yes	36	8
	No	17	19
Tumor site	Laryngeal	33	27
	Hypopharyngeal	25	0
Differentiation	High	29	-
	Low	29	-
Stage	I	13	-
	II	10	-
	III	13	-
	IV	21	-
Lymph node metastasis	Yes	25	-
	No	32	-
P53	+	35	-
	-	23	-
P16	+	5	-
	-	53	-
P63	+	33	-
	-	25	-
Neoadjuvant therapy	Yes	11	-
	No	47	-

Table 2 Correlation between serum cytokine levels and different diseases.

	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-17	TNF-α	IFN-α	IFN-γ
Tumor (Median)	5.585	2.63	1.735	3.545	5.56	3.945	1.59	2.47	3.77	2.875	2.9	6.205
Benign (Median)	5.46	3.08	3.12	3.65	4.21	3.22	2.67	2.82	4.51	4.01	3.32	5.69
P	0.6296	0.0510	0.0130	0.8933	0.0213	0.4447	0.0399	0.0466	0.0678	0.0297	0.2158	0.6515

Table 3. Correlation between serum cytokine levels and disease characteristics.

		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-17	IL-12p70	TNF-α	IFN-α	IFN-γ
Differentiation	High Differentiation	3.51	2.815	2.08	3.64	4.34	2.635	2.005	2.47	3.685	3.56	2.87	5.015
	Low Differentiation	9.15	1.5	1.68	3.53	8.16	4.61	1.48	2.16	4.11	2.35	3.23	6.79
	P	0.0078	0.288	0.8045	0.4654	0.0045	0.0496	0.7435	0.4579	0.4579	0.5873	0.5268	0.6491
Tumor site	Laryngeal	6.2	2.365	1.49	3.63	5.835	2.85	1.255	1.965	3.32	2.87	3.095	5.015
	Hypopharyngeal	5.46	2.73	2.41	3.49	4.93	4.24	2.27	2.65	4.11	3.72	2.76	6.47
	P	0.8844	0.7112	0.0479	0.6581	0.7316	0.2469	0.2921	0.403	0.2996	0.3109	0.4676	0.6237
Lymph node metastasis	Yes	3.895	1.53	1.145	3.51	5.115	2.455	1.21	1.535	2.765	1.44	2.785	4.965
	No	8.13	3.17	2.715	3.66	5.97	6.19	2.555	2.735	4.5	4.39	3.295	11.06
	P	0.0755	0.0247	0.0049	0.1823	0.5933	0.0093	0.0167	0.0017	0.0002	0.0003	0.2727	0.0259

Table 4. Correlation analysis between the efficacy of neoadjuvant therapy and changes in serum cytokine levels.

Grouping		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-17	IL-12p70	TNF-α	IFN-α	IFN-γ
PR	Before (Median)	8.13	2.85	1.87	3.64	5.1	6.06	2.56	2.74	4.87	3.15	3.41	12.8
	After (Median)	8.13	3.2	3.31	4.64	4.69	3.99	2.79	2.87	4.59	3.74	3.06	20.2
	P	0.5781	0.0156	0.0156	0.8125	0.375	0.6875	0.0313	0.2188	0.271	0.4688	0.1563	0.0156
		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-17	IL-12p70	TNF-α	IFN-α	IFN-γ
SD	Before (Median)	4.57	0.49	2.32	3.52	7.1	2.86	1.88	2.17	4.83	2.9	2.34	6.49
	After (Median)	3.6	3.24	2.84	3.48	10.8	3.83	2.69	2.76	4.6	4	2.95	8.6
	P	0.875	0.125	0.625	1	0.625	0.875	0.625	0.875	0.875	0.625	0.625	0.625

Table 5. Correlation analysis between serum cytokines and markers.

		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-17	TNF-α	IFN-α	IFN-γ
P16	Z	-1.738	-3.064	-1.746	-0.931	-2.092	-1.371	-2.286	-2.036	-1.316	-2.037	-3.267	-1.275
	P	0.082	0.002	0.081	0.352	0.036	0.17	0.022	0.042	0.188	0.042	0.001	0.202
P63	Z	-0.999	-2.062	-1,113	-0.814	-0.063	-0.908	-1.224	-0.513	-0.979	-1.547	-1.473	-0.513
	P	0.318	0.039	0.266	0.416	0.95	0.364	0.221	0.608	0.328	0.122	0.141	0.608
Ki-67	Z	0.048	0.013	0.141	0.093	-0.037	0.291	0.098	0.068	0.098	-0.02	0.161	0.203
	P	0.721	0.925	0.295	0.495	0.783	0.028	0.468	0.616	0.468	0.883	0.23	0.13
PD-L1(CPS)	Z	0.157	0.115	0.106	-0.094	0.279	0.108	0.085	0.118	-0.081	0.152	0.433	0.333
	P	0.389	0.533	0.564	0.616	0.122	0.557	0.645	0.522	0.658	0.406	0.013	0.062