Increased prevalence of $T_{H17}$ cells in the peripheral blood of patients with head and neck squamous cell carcinoma

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Objective. This study was performed to determine whether $T_{H17}$ cells are involved in the development and metastasis of head and neck squamous cell carcinomas (HNSCCs).

Study design. $T_{H17}$ cells frequencies in 67 HNSCC patients and 21 healthy volunteers were examined by flow cytometric analysis. $T_{H17}$ cell–related cytokines in serum (interleukin (IL) 17, transforming growth factor (TGF) β, and IL-6) were evaluated by using enzyme-linked immunosorbent assay.

Results. It was discovered that the higher frequency of $T_{H17}$ cells was in HNSCCs patients (1.0 ± 0.4%). The cell proportions and related cytokine concentrations were consistent with the tumor TNM stage. The IL-6 concentration showed positive correlation with the frequency of $T_{H17}$ cells ($r = 0.661$) and IL-17 levels ($r = 0.597$). The TGF-β concentration showed a positive correlation with IL-17 ($r = 0.626$) but no relationship with $T_{H17}$ cells ($r = 0.431$).

Conclusions. The present data suggested that $T_{H17}$ cells may be involved in tumor growth and metastasis of HNSCCs. IL-6 may play an important role in $T_{H17}$ cell differentiation and functions, and TGF-β may be related to IL-17 secretion but not to the differentiation of $T_{H17}$ cells. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:81-89)

Squamous cell carcinoma is the most common malignancy in the head and neck region. Although treatment methods such as surgery, chemotherapy, and radiotherapy, or a combination of all 3 therapies, have been used for patients with head and neck squamous cell carcinomas (HNSCCs), recurrence and metastasis could often be detected after treatment of the primary tumor and remain the most frequent cause of mortality, especially in patients with advanced tumors.1,2 Recently, with advances in cellular and molecular immunology, much attention has been focused on the interaction of the immune system and HNSCC tumor cells.

$T_{H17}$ cells, the third subset of CD4+ T helper cells (T lymphocytes that belong to the CD4+ subset), which are characterized by their production of interleukin (IL) 17A and IL-17F, were first identified in 2005. $T_{H17}$ cells have been verified to play an important role in host defense against extracellular microbial infection, which $T_{H1}$ and $T_{H2}$ cells are not well suited for.3 These cells follow a differentiation pathway distinct from $T_{H1}$ and $T_{H2}$ cells, requiring the combination of transforming growth factor (TGF) β and IL-6. Several lines of evidence suggest that $T_{H17}$ cells contribute to inflammation, autoimmune diseases, and human organ transplantation rejection.4-8

Recent investigations have reported an increase in $T_{H17}$ cells numbers observed in malignant tumors, such as gastric cancer, epithelial ovarian carcinoma, renal cell carcinoma, and pancreatic carcinoma.9,10 These findings suggest that $T_{H17}$ cells may play an important role in tumor immunoeediting and pathogenesis. Therefore, we examined the proportion of $T_{H17}$ cells in peripheral blood and the concentration of $T_{H17}$ cell–related factors (IL-17, TGF-β, and IL-6) in the serum of patients with HNSCCs to investigate whether $T_{H17}$ cells are involved in tumor progression and metastasis.
MATERIAL AND METHODS

Patients

The study comprised 67 patients with HNSCC hospitalized at the Department of Oral and Maxillofacial Surgery, Stomatologic Hospital of Wuhan University from January 2008 to March 2009. Ten mL of peripheral blood was collected from each of the patients and 21 healthy volunteers. The patients included 36 men and 31 women, aged from 31 to 79 years, with a mean age of 61.8 years. Patients who smoked >10 cigarettes per day and/or drank >3 drinks per day were excluded from the study. The gender, average age, smoking, and drinking habits of volunteers were similar to the patients. Those with autoimmune and inflammatory disease (patients with acute or chronic inflammation of respiratory system, urinary system, alimentary system) were excluded from the study. Patients and volunteers with severe periodontitis and lichen planus also were excluded. None of the patients received chemotherapy or radiotherapy before the blood samples were taken. Patients who received antibacterial therapy >1 week were excluded. The primary sites of carcinomas were tongue,28 buccal mucosa,15 gingiva,12 and floor of the mouth.12 TNM clinical stage was evaluated according to the version established by the International Union Against Cancer (UICC): stage I = T1N0M0; stage II = T2N0M0; stage III = T3N0M0, or T1, T2, or T3N1M0; stage IV = any T4, N2, N3, or M1 lesion (Table I).

Blood samples

Blood samples were obtained from all the patients on the morning following admission. All of the samples from patients and volunteers were collected into collection tubes containing 2% sodium citrate. Peripheral blood mononuclear cells (PBMCs) were prepared by standard Ficoll-Hypaque density centrifugation for flow cytometric analysis. Each blood sample was divided into 2 parts as PBMCs for flow cytometric analysis of TH17 cells and serum for enzyme-linked immunosorbent assay (ELISA).

Flow cytometric analysis of T_{h}17 cells

PBMCs were suspended at a density of $2 \times 10^6$ cells/mL in RPMI1640 complete culture medium supplemented with 100 U/mL penicillin, 1 µg/mL streptomycin, 2 mmol/L glutamine, and 10% heat-inactivated fetal calf serum. The cell suspension was transferred to 96-well plates. Cells were stimulated with 50 ng/mL phorbol myristate acetate plus 1 µmol/L ionomycin and 1.7 µg/mL monensin, then incubated at 37°C under a 5% CO₂ environment for 4 hours. The cells were aliquoted into 5-mL sterile tubes, washed twice in phosphate-buffered saline solution (PBS), then centrifuged at 1,500 rpm for 5 minutes. For T_{h}17 cell analysis, the cells were incubated with phycoerythrin (PE) antihuman CD4 at 4°C for 20 minutes. After the surface staining, the cells were stained with fluorescein isothiocyanate–antihuman IL-17A for T_{h}17 detection after fixation and permeabilization according to the manufacturer’s instructions. All the antibodies were from eBioscience, San Diego, CA, USA. Isotype controls were given to enable correct compensation and confirm antibody specificity. Stained cells were then analyzed by BD LSRII flow cytometer.

Enzyme-linked immunosorbent assay

Peripheral blood of patients with HNSCC and healthy volunteers samples were kept to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2,000 rpm. We removed serum immediately and stored samples at −20°C. Serum levels of IL-17, IL-6, and TGF-β were determined by using specific ELISA kits (eBioscience) according to the manufacturer’s recommendation and previous report.11 All samples were measured by duplicate. We incubated each ELISA plate with 100 µL captured antibody overnight at 4°C. The wells were washed 5 times with >250 µL/well wash buffer. We diluted 5× concentrated assay diluent with deionization (DI) water and incubated it 1 hour at room temperature. We added serum samples (diluted 1:20 in PBS containing 25% FBS) to the wells at the concentration of 100 µL/well and incubated them at room temperature for 2 hours followed by addition of 100 µL.

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**Table I. Clinical characteristics (n) of patients with head and neck squamous cell carcinomas (HNSCCs)**

<table>
<thead>
<tr>
<th>Age</th>
<th>51.2 ± 14.7 y</th>
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<tbody>
<tr>
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<tr>
<td>Female</td>
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<td>Tumor site</td>
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<td>Tongue</td>
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<tr>
<td>Buccal mucosa</td>
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<tr>
<td>Gingiva</td>
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<tr>
<td>Floor of mouth</td>
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<tr>
<td>Tumor size</td>
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<tr>
<td>T1</td>
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<td>T2</td>
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<td>T3</td>
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<td>T4</td>
<td>9</td>
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<tr>
<td>Lymph node</td>
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<tr>
<td>N0</td>
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<tr>
<td>N1</td>
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<tr>
<td>N2</td>
<td>15</td>
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<td>III</td>
<td>21</td>
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<tr>
<td>IV</td>
<td>19</td>
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of detection antibody (0.25 μg/mL) for 1 hours. Then, added 100 μL/well of Avidin-HRP diluted in 1× Assay Diluent. Wells were washed 4× with PBS containing 0.05% Tween 20 between each step. We then added 100 μL substrate solution to each well and incubated the plates at room temperature for 15 minutes. We added 50 μL stop solution to each well and read the plates by Tecan Sunrise Absorbance Reader at 450 nm.

Statistical analysis
All data were analyzed with SPSS 15.0 software. Data are presented as mean ± SD. Comparison between 2 groups was performed by Student t test. Variation among independent groups were determined by one-way analysis of variance. P < .05 was considered to be statistically significant. Correlations between variable cytokines were determined by Pearson correlation; r > 0.5, P < .05 was considered to be positive correlation.

RESULTS

**T_{H17} cell proportion in peripheral blood of patients with HNSCCs**

We detected T_{H17} cell (CD4^+ IL-17^+ T cells) proportions in peripheral blood of 67 HNSCC patients and 21 healthy volunteers by flow cytometric analysis. T_{H17} cell proportion in peripheral blood of patients with HNSCCs was 1.0 ± 0.4% and in healthy controls was 0.3 ± 0.1% (P < .05). The prevalence of T_{H17} cells in stage I, II, III, and IV was 0.5 ± 0.2%, 0.8 ± 0.4%, 1.1 ± 0.5%, and 1.9 ± 0.6%, respectively, with significant differences among the groups (P < .05; Fig. 1). The results clearly showed that T_{H17} cell frequencies increased in a very early stage of tumor. The proportion significantly increased in patients with advanced HNSCC compared with early stage of tumor as well as normal control subjects. T_{H17} cell proportion in T1, T2, T3, and T4 was 0.5 ± 0.2%, 0.9 ± 0.3%, 1.4 ± 0.5%, and 1.5 ± 0.6%, respectively, with statistical difference (P < .05), but no differences could be detected between patients with T3 and T4 tumor. There were significant differences between T_{H17} cell proportions in peripheral blood of HNSCC patients with or without lymph node metastasis (1.4 ± 0.6% vs .70 ± 0.3%; P < .05; Fig. 2).

**Serum levels of IL-17 in HNSCC patients and healthy control subjects**

To visualize the expression of IL-17 in the peripheral blood of HNSCC patients, we performed ELISA to detect this cytokine in the serum of 67 HNSCC patients and 21 healthy volunteers. The concentrations of IL-17 are presented in Table II. The serum levels of IL-17 increased in patients with HNSCCs compared with healthy control subjects (123.35 ± 45.13 pg/mL vs. 20.78 ± 3.95 pg/mL; P < .05). Concentration of IL-17 was 56.63 ± 21.51 pg/mL in stage I, 112.89 ± 45.31 pg/mL in stage II, 125.99 ± 50.26 pg/mL in stage III, and 160.45 ± 48.07 pg/mL in stage IV (P < .05). But there were no significant differences between the concentration of IL-17 in stages II and III (Fig. 3, a). High level of IL-17 was found in HNSCC patients with metastasis as 145.21 ± 55.86 pg/mL, whereas IL-17 level was 99.78 pg/mL in patients without metastasis (Fig. 4, a).

**Serum levels of IL-6 and TGF-β in HNSCC patients and healthy control subjects**

The serum concentrations of TGF-β was significantly higher in HNSCC patients than in healthy control subjects (215.80 ± 51.53 pg/mL vs. 26.96 ± 10.44 pg/mL; P < .05; Table II). The concentration of TGF-β in stages I, II, III, and IV was 62.98 ± 18.00 pg/mL, 170.44 ± 56.93 pg/mL, 193.36 ± 80.79 pg/mL, and 339.46 ± 75.47 pg/mL, respectively (Fig. 3, b), with significant difference between the early stage of tumor and advanced tumor (P < .05) but no significant difference between TGF-β concentration in patients with stages II and III tumor. The level of TGF-β in patients with lymph node metastasis was higher than without metastasis (270.49 ± 109.17 pg/mL vs. 150.89 ± 45.97 pg/mL; P < .05; Fig. 4, b).

The IL-6 levels also increased during the development of HNSCCs. The concentration of IL-6 was 5.85 ± 1.48 pg/mL in healthy control subjects, compared with 17.32 ± 8.03 pg/mL in patients with HNSCCs (P < .05). Concentration of serum IL-6 in stages I, II, III, and IV were 9.81 ± 4.25 pg/mL, 15.49 ± 3.81 pg/mL, 16.99 ± 7.28 pg/mL, and 25.65 ± 6.62 pg/mL, respectively (Fig. 3, c), the variation between the early stage of tumor and advanced tumor had statistical significance (P < .05), but no significant difference could be detected between IL-6 concentration in patients with stages II and III of tumor. There were significant differences between concentration of IL-6 in peripheral blood of HNSCC patients with or without lymph node metastasis (21.49 ± 8.20 pg/mL vs. 14.82 ± 6.99 pg/mL; P < .05; Fig. 4c).

**Correlation between populations of T_{H17} cells in the peripheral blood and serum IL-17 levels with TGF-β and IL-6**

Pearson correlation was used to determine the associations of TGF-β and IL-6 with populations of peripheral blood T_{H17} cells and serum IL-17 concentrations, respectively, in patients with HNSCCs. No positive correlation between TGF-β and T_{H17} cell frequencies could be detected (Fig. 5, a; r = 0.431; P > .05). Correlation analysis showed positive correlation between TGF-β and IL-17 (Fig. 6, a; r = 0.626; P < .05).
The serum IL-6 concentrations showed a positive correlation with T\textsubscript{H}17 cell frequencies (Fig. 5, b; $r = 0.661; P < .05$) and IL-17 concentrations (Fig. 6, b; $r = 0.597, P < .05$).

**DISCUSSION**

T-Cell–based immunotherapy for malignant tumors has recently attracted increasing attention, because conventional therapy, such as surgery, radiotherapy, and chemotherapy, has limited efficacy. Investigation into the interactions of the host immune system and tumors could lead to better understanding of tumor immunotherapy and more accurate clinical testing.

The concept of immunoediting describes the role of the immune system in tumor pathology and development. It includes 3 phases: elimination of tumor cells, equilibrium between the developing tumor and the immune system, and tumor escape. Accumulating evidence suggests that the immune system may either protect the host against tumor or promote tumor aggressiveness. In 69 patients with muscle-invasive urothelial carcinoma, high numbers of tumor-infiltrating T cells (TILs) were detected in 61% of patients with very early stage of tumors. The disease-free survival and overall survival of patients with CD8\textsuperscript{+} TILs was much longer than those whose tumors did not express CD8\textsuperscript{+} TILs. CD8\textsuperscript{+} and CD4\textsuperscript{+} TILs were found in 63.9% and 71.3% of patients with melanoma, respectively, correlating with the positive expression of major histocompatibility complex class I complex.
models also demonstrate the indirect antitumor effect of CD4+ T cells mediated by Th1 and Th2 cell–related cytokines. CD4+ T cells within the microenvironment of human nonsmall cell lung carcinoma may secret interferon-γ to inhibit tumor cell proliferation and induced tumor regression.

Earlier studies have demonstrated that Th17 cells are involved in tissue damage of many autoimmune and inflammatory diseases and allograft rejection. Most of the biologic roles of Th17 cells in the pathogenesis of disease are mediated by IL-17, the cytokine secreted by Th17 cells. Recently, involvement of Th17 cells in tumor development has been elucidated in animal models and human tumors. Kryczek et al. first examined Th17 cells in human subjects with cancer. Th17 cells numbers were significantly up-regulated in the peripheral blood and tumor tissues of patients with epithelial ovarian carcinoma, renal cell carcinoma and pancreatic carcinoma. Furthermore, Muranski et al. reported that Th17-polarized cells could eradicate large established melanomas, suggesting that Th17 cells may be involved in adoptive immunotherapy for human malignant tumors. An ovalbumin-specific Th17 cell adopted transfer experiment showed a significantly increased number of CD45+ CD4+, and CD8+ leukocytes, whereas reduced tumor colonies suggested a tumor-protective effect of Th17 cells.

In the present study, the proportion of Th17 cells in the peripheral blood of patients with HNSCCs was

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Table II. Levels (pg/mL) of interleukin (IL) 17, transforming growth factor (TGF) β, and IL-6 in the serum of healthy donors and patients with head and neck squamous cell carcinomas (HNSCCs)

<table>
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<th>IL-17</th>
<th>TGF-β</th>
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<tr>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>I</td>
<td>56.63 ± 21.51</td>
<td>62.98 ± 18.00</td>
<td>9.81 ± 4.25</td>
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<tr>
<td>II</td>
<td>112.89 ± 45.31</td>
<td>170.44 ± 56.93</td>
<td>15.49 ± 3.81</td>
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<tr>
<td>III</td>
<td>125.99 ± 50.26</td>
<td>193.36 ± 80.79</td>
<td>16.99 ± 7.28</td>
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<tr>
<td>IV</td>
<td>160.45 ± 48.07</td>
<td>339.46 ± 75.47</td>
<td>25.65 ± 6.62</td>
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<tr>
<td>Lymph node metastasis</td>
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<tr>
<td>Negative</td>
<td>99.78 ± 36.87</td>
<td>150.89 ± 45.97</td>
<td>14.82 ± 6.99</td>
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<tr>
<td>Positive</td>
<td>145.21 ± 55.86</td>
<td>270.49 ± 109.17</td>
<td>21.49 ± 8.20</td>
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</table>
The frequencies of TH17 cells were higher in patients with advanced tumors (stages III and IV) than those in patients with an early stage of tumor. Significant differences were detected in the frequencies of TH17 cells between T categories (P < .05). Patients in T3 and T4 had a higher percentage of circulating TH17 cells than those in T1 and T2; in addition, we found the highest frequencies of TH17 cells in patients with lymph node metastasis. Our results suggested that TH17 cells in the peripheral circulation appear to be accompanied by cancer growth and metastasis.

As the most important cytokine secreted by TH17 cells, IL-17 may play a potential role in tumor growth. Several reports have investigated the effects of IL-17 in the development of malignant tumors. The CD4+ T-cell–derived cytokine IL-17 enhanced tumor angiogenesis by inducing the migration of endothelial cells and significantly promoting the formation of new endothelial cords. IL-17 up-regulated angiogenic factors and angiogenic cytokines, such as vascular endothelial growth factor, keratinocyte-derived chemokine, and prostaglandin E2, and indirectly promoted the growth of the murine adenocarcinoma cell line. Earlier data also showed a tumor-promoting activity of IL-17 in human cervical cancers by inducing the recruitment of macrophages to the tumor site and enhancing IL-6 secretion. In contrast, some reports also suggest that IL-17 has an antitumor activity. Benchetrit et al. reported an antitumor activity of IL-17 through a host-
dependent mechanism by enhancing the generation of tumor cell–specific cytotoxic T lymphocytes in immunocompetent mice models. These opposite data of pro-tumor or antitumor effect by IL-17 suggest that the activity of this cytokine may depend on the immunogenicity of the tumor. In the present study, we evaluated the concentration of IL-17 in the serum of patients with HNSCCs and in healthy volunteers by ELISA.

Fig. 5. Pearson correlation of Th17 cell frequencies in the peripheral blood and transforming growth factor (TGF) β and interleukin (IL) 6 levels in serum of head and neck squamous cell carcinoma (HNSCC) patients. a, TGF-β concentrations showed no positive correlation with Th17 cell frequencies \( (r = 0.431; \ P > 0.05) \). b, IL-6 concentrations show positive correlation with Th17 cell frequencies \( (r = 0.661; \ P < 0.05) \).

Fig. 6. Pearson correlation of interleukin (IL) 17 levels and transforming growth factor (TGF) β and IL-6 levels in serum. a, TGF-β concentrations showed positive correlation with IL-17 levels \( (r = 0.626; \ P < 0.05) \). b, IL-6 concentrations showed positive correlation with IL-17 levels \( (r = 0.597; \ P < 0.05) \).
The results clearly indicated that IL-17 expression can be detected in the very early stage of squamous cell carcinoma and increased gradually with the development of the tumor. The concentration of this TH17-related cytokine coincided with the increased number and proportion of TH17 cells in the peripheral blood of patients with HNSCCs. These results suggested that IL-17 may be involved in the pathogenesis of HNSCCs.

The development and differentiation of TH17 cells is a complex process requiring the action of many cytokines. Studies in mice suggested that IL-6 and TGF-β were the key regulators in TH17 differentiation. IL-6 plus TGF-β may effectively induce the development of TH17 cells in the peripheral blood of mice. However, the development and differentiation of human TH17 cells seem to be through a distinct pathway compared with the same mechanism in mice, and the roles of IL-6 and TGF-β in the generation of human TH17 cells are still under debate. Early reports revealed that a combination of IL-21 and IL-6 is required for the differentiation of TH17 cells, and IL-6 and TGF-β alone or TGF-β plus IL-6 failed to induce the differentiation of human TH17 cells. However, recent studies have shown that the independence of TGF-β may be due to the culture medium of human naive CD4+ T cells, which already contain small doses of TGF-β. TGF-β alone was necessary to up-regulate the differentiation of TH17 cells but failed to induce IL-17 expression in human naive CD4+ T cells in a serum-free condition, whereas TGF-β plus IL-6 was able to induce IL-17 secretion sufficiently. TGF-β uniquely induced TH17 cell differentiation and IL-17A secretion by inducing high levels of retinoic acid receptor-related orphan receptor C2, a critical transcription factor for human TH17 cell differentiation. However, IL-6 alone had a limited effect on IL-17 induction.

Our data indicated that increased expressions of TGF-β and IL-6 were consistent with the tumor progression. The concentrations of these 2 cytokines increased in patients with advanced tumors more than with early stages of disease. Both of the 2 cytokines were higher in patients with lymph node metastasis. Serum concentrations of IL-6 showed a correlation with both the proportions of TH17 cells and the IL-17 levels in patients with HNSCCs. TH17 cell frequencies and IL-17 levels elevated with the increase in TGF-β concentration, and there was a positive correlation between TGF-β and IL-17 concentrations in serum. However, the concentrations of TGF-β did not show positive correlation with TH17 cell frequencies. These results were consistent with Wu et al.’s report which indicated that IL-6, but not TGF-β, was involved in TH17 cell differentiation in patients with acute myeloid leukemia. The results suggested that TGF-β and IL-6 may participate in IL-17 secretion in circulation. IL-6 may play an important role in TH17 cell differentiation, and the role of TGF-β in the differentiation of TH17 cells in HNSCC patients still requires further investigation.

In conclusion, our study indicated that the proportion and activity of TH17 cells increased in the circulation of patients with HNSCCs and were consistent with tumor progression. TH17 cells might be involved in tumor growth and metastasis of HNSCCs. IL-6 may play an important role in TH17 differentiation and function, and TGF-β may be involved only in IL-17 secretion. The regulation of the number and function of TH17 cells may help us to better understand the tumor immunity in patients with HNSCCs and provide new insight into more effective immunotherapy for this type of tumor.

REFERENCES


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