Neutrophils Promote Larynx Squamous Cell Carcinoma Progression via Activating the IL-17/JAK/STAT3 Pathway

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1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is generated from the laryngeal mucosal epithelium. LSCC accounts for 2.4% of systemic malignancies and more than 90% of laryngeal carcinoma [1]. More than 90,000 people died of LSCC every year. LSCC patients suffer from severe impairments in breathing and swallowing, which cause great pain. More than half of the patients are diagnosed in an advanced stage [2]. LSCC patients’ survival is seriously affected by local invasion and metastasis [3]. Therefore, it is of great importance to figure out the mechanism of LSCC progression and find new targets for therapy.

One of the important causes of LSCC patients’ poor survival is metastasis [4]. However, the precise mechanisms leading to the metastasis remain unclear [5, 6]. Increasing evidences show that the microenvironment plays important roles in tumor progression [7, 8]. Immune cells, fibroblasts, and mesenchymal stem cells are important components of microenvironments [9]. Until recently, immune cells, especially macrophages, highlight the cancer research. However, with the development of new technologies, more and more functions of neutrophils in cancer are reported.

Neutrophils are the most prevalent type of immune cells, which are the first responder cells to various inflammations and infections [10]. Neutrophils are reported to play important functions through every step of cancer progression, including initiation, growth, and metastasis [11]. Neutrophils could play diverse and even opposite functions in tumor progression [11–13]. Increasing evidence suggests that tumor-associated neutrophils could both promote and inhibit cancer progression [14, 15]. The neutrophil-to-lymphocyte ratio has been confirmed to be a predictor for LSCC poor survival [16, 17]. And tumor-infiltrating neutrophils are also correlated with poor LSCC prognosis [18]. But the specific mechanism of how neutrophils regulate prognosis of LSCC remains unclear. In this research, we want to investigate the crosstalk between neutrophils and LSCC.
### 2. Materials and Methods

#### 2.1. Cell Culture.

Tu177, Tu686, and HL-60 were maintained as described by previous reports [17, 19, 20]. Tu177 was obtained from Qincheng Biological Co., Ltd. Tu686 was from Tongpai Biological Technology Co., Ltd. HL-60 cells were obtained from ATCC (the Global Bioresource Center). Cells were cultured with RPMI-1640 medium containing 100 IU/mL of penicillin, 100 \( \mu \)g/mL of streptomycin, and 10% of FBS. All the cells were cultured with 5% CO\(_2\) at 37\(^\circ\)C.

#### 2.2. Conditioned Medium from Neutrophils.

HL-60 cells were seeded into cell culture dishes at the density of \(4 \times 10^3\) cells/mL. HL-60 cells were treated with 1.25% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) for five days to form neutrophils. The control group was cultured without DMSO for five days.

At the sixth day, the neutrophils were cultured with fresh 1640 medium with 10% FBS and cultured for 24 hours at 37\(^\circ\)C with 5% CO\(_2\). The cells and supernatants were separated by centrifugation at 2000g/min for 15 min. We collected the supernatants as the conditioned medium from neutrophils.

#### 2.3. Conditioned Medium from LSCC Cells.

Tu177 and Tu686 were seeded onto cell culture dishes at the density of \(1 \times 10^4\) cells/mL. Cells were cultured with 1640 medium with 10% FBS (Gibco, Invitrogen, USA) for 24 hours. The cells and supernatants were separated by centrifugation at 2000g/min for 15 min. We collected the supernatants as the conditioned medium from neutrophils.

#### 2.4. Western Blot.

Cells are lysed with NETN150 (0.5% NP-40, 20 mM Tris (pH 8.0), 150 mM NaCl, and 6 mM EDTA). 25 \( \mu \)g of proteins was separated by a 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred to NC membranes and blocked with 5% nonfat milk. Then, we incubated the membranes with the indicated primary antibody. Protein levels were detected by western blot as previously described [21].

#### 2.5. qRT-PCR.

A TRIzol kit (Invitrogen, NY) was applied for RNA extraction following the instruction of the manufacturer. A reverse transcription kit (Invitrogen) was used for RNA reverse transcription. A SYBR kit (Roche) was used for qRT-PCR. The sequences of genes are shown in Table 1.

#### 2.6. Proliferation Assay.

4000 cells were seeded into 96-well plates per well. The cells were cultured with complete medium (control) or conditioned medium from neutrophils. The conditioned medium from neutrophils was added with or without the IL-17 antibody at the concentration of 200 \( \mu \)M. We used the CCK8 kit (Dojindo, Kumamoto, Japan) for proliferation assays. We detected the absorbance at 450 nm at day 0, day 2, day 4, and day 6 following instructions of the manufacturer.

#### 2.7. ELISA.

IL-17 concentration was detected by using an ELISA kit (R&D Systems, USA) under the direction of the manufacturer’s instruction.

#### 2.8. Migration for Neutrophils.

1:2×10\(^5\) HL-60-induced neutrophils were plated onto the upper chambers. Neutrophils which migrated into the lower chambers were collected and counted by using the Bio-Rad TC10 automatic cell counter 8 hours later. Each data was performed in triplicate, and the experiments were independently repeated three times.

#### 2.9. Migration and Invasion Assays for LSCC Cells.

24-well chambers (Corning, CA, USA) were used. 2 \( \times 10^4\) cells with medium without FBS were added into the upper chamber. The down chambers were filled with conditioned medium or complete medium (control). The conditioned medium from neutrophils was added with or without 1 \( \mu \)M IL-17 antibody (Novartis Cosentyx). The chambers were cultured at 37\(^\circ\)C for 24 hours. The staining of the cells was performed as previously reported [15]. The chambers were pretreated with Matrigel for the invasion assay [15].

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**Table 1**

| Gene   | Forward primer | Reverse primer |
|--------|----------------|----------------|
| TGF-\(\beta\) | GGCCAGATCTCGTCAAGGC | GTGGGTTCACACTTAGCAC |
| IL-1   | ATGATGGCTTATTACAGTGGCA | GTGGGTTCACACTTAGCAC |
| G-CSF  | TCCCTGAACCTCGGACAGAAC | GTGGGTTCACACTTAGCAC |
| IL-4   | GCAAGACCCCTCAGAACAGAAA | GTGGGTTCACACTTAGCAC |
| IL-6   | ACTGACCTTCGAGAAACCATTAG | GTGGGTTCACACTTAGCAC |
| IL-8   | TTTTGGACAAAGATGCTAAGAAG | GTGGGTTCACACTTAGCAC |
| IL-10  | GACCTTAAAGGTGTACGACATGC | GTGGGTTCACACTTAGCAC |
| IL-12  | ACCCTGACCATCAGAATCCAA | GTGGGTTCACACTTAGCAC |
| IL-15  | TTTTGGAAACATAGATTTGTCAG | GTGGGTTCACACTTAGCAC |
| IL-17  | TCCCAGGAAATCAGGAGATAC | GTGGGTTCACACTTAGCAC |
| IL-20  | ATGAAGGCTACGTTGCCTGCT | GTGGGTTCACACTTAGCAC |
| IL-23  | CTCAGGGACAAACTGATTGTTC | GTGGGTTCACACTTAGCAC |
| GAPDH  | CTGGGCTCACTGAGACCAC | GTGGGTTCACACTTAGCAC |

qRT-PCR results were calculated by the method of \(2^{\Delta\Delta Ct}\), and glyceral-dehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference. The indicated gene expression was normalized to GAPDH.
Figure 1: Continued.
2.10. Drugs and Antibodies. The STAT3 inhibitor SH-4-54 was obtained from Selleck Company. Antibodies for vimentin, Snail, E-cadherin, p-JAK, JAK, p-STAT3, STAT3, and β-actin were all obtained from Cell Signaling Technology (Louis Park, MN, USA). The IL-17 antibody was from Novartis Cosentyx. Recombinant human IL-17 was from Sigma-Aldrich.

2.11. Statistical Analyses. All analyses were performed by using GraphPad Prism 8.0. Data were shown as mean ± S.D. The difference was determined by Student’s t-test and analysis of variance. P < 0.05 was identified as statistically significant.

3. Results

3.1. LSCC Cells Enhance Neutrophil Activation and Migration. Neutrophils, as one of the most abundant immune cells, have been reported to play important roles in cancer progression. Firstly, we detected the influence of LSCC cells on neutrophils. The spatial and temporal regulations of β2 integrin CD11b/CD18 and Myeloperoxidase (MPO) are reported to play essential functions in activation and recruitment of neutrophils [22]. Signaling via adhesion molecules of the beta2 integrin family (CD11/CD18) plays important roles in polymorphonuclear leukocyte (PMN) recruitment and activation during inflammation [23]. MPO catalyzes the formation of reactive oxygen intermediates, including hypochlorous acid (HOCl). The MPO/HOCl plays an important role in microbial killing by neutrophils [24]. The neutrophils were cocultured with RPMI-1640 medium with 10% FBS, RPMI-1640 with 1.25% DMSO and 10% FBS, conditioned medium of Tu177 and Tu686, and conditioned medium of Tu177 and Tu686 with 1.25% DMSO, respectively. qRT-PCR analysis revealed that the conditioned medium of Tu177 and Tu686 could significantly upregulate CD11b, CD18, and MPO levels compared with the control group (Figures 1(a)–1(g)). These results suggested that LSCC conditioned medium could enhance

![Figure 1](https://example.com/figure1.png)
Cancer cells
Neutrophils conditioned medium
Day0 Day2 Day4 Day6
Relative growth rate
Tu177 growthrate
CTL Neutrophils CM
Tu686 growthrate
CTL Neutrophils CM
LSCC cells
Medium without FBS
Neutrophils conditioned medium
Tu177 migration
Tu686 migration
Figure 2: Continued.
the neutrophil activation. We also detected the influence of LSCC conditioned medium on neutrophil mobility. Transwell assays were used to detect the neutrophil mobility. 1640 with 10% FBS (control medium) or LSCC conditioned medium was added to the lower chamber. Transwell assays also indicated that the conditioned medium of Tu177 and Tu686 could promote the migration of neutrophils than the control medium (Figures 1(h)–1(j)). Together, LSCC cells could promote the activation and migration of neutrophils without direct interaction.

3.2. Neutrophils Promote Proliferation, Migration, and Invasion of LSCC. Functions of neutrophils on LSCC were explored by the proliferation assay, migration assay, and invasion assay. LSCC cells were cocultured with neutrophil conditioned medium, and proliferation rates were detected by CCK8. The results showed that neutrophil conditioned medium promoted the proliferation rates of both Tu177 and Tu686 cells (Figures 2(a)–2(c)). LSCC cells were added to the upper chamber. Conditioned medium was added to the lower chamber. The chambers were cultured at 37°C for 24 hours. (e, f) Migration results of Tu177 and Tu686 cells. (g, h) Invasion results of Tu177 and Tu686 cells.

3.3. IL-17 Is Responsible for LSCC Progression Induced by Neutrophils. Increasing amounts of evidence show that neutrophils could promote cancer progression through inflammatory factors [25]. We detected the levels of inflammatory factors in neutrophil conditioned medium. Results showed that the conditioned medium contained INFβ, TGFβ, G-CSF, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, IL-15, IL-17, IL-20, and IL-23 (Figure 3(a)). ELISA also confirmed the expression of IL-8, IL-10, IL-12, and IL-17 (Figure 3(b)). All results suggested that IL-17 was highly expressed in neutrophil conditioned medium.

To confirm whether IL-17 is the main factor contributing to the LSCC progression, we used the IL-17 antibody (Novartis Cosentyx) to block the function of IL-17. CCK8 assays showed that blockage of IL-17 could effectively weaken the proliferation rates of Tu177 and Tu686, which were enhanced by neutrophil conditioned medium (Figures 3(c) and 3(d)). Further studies also showed that IL-17 blockage could recede the migration and invasion ability induced by neutrophils (Figures 3(e)–3(h)). What is more, western blot results showed that neutrophils could promote the EMT (Epithelial-Mesenchymal Transition) of Tu177 and Tu686, and blockage of IL-17 could effectively inhibit the EMT (Figures 3(i) and 3(j)). All these experiments confirmed that IL-17 was responsible for neutrophil-induced LSCC progression.

3.4. Neutrophils Activate the JAK/STAT3 Pathway in LSCC Cells. JAK/STAT3 (the Janus kinase/signal transducer and activator of transcription 3) pathway activation has been found in various cancers. STAT3 (signal transducer and activator of transcription 3) is also reported to play important roles in LSCC chemoresistance, growth, and mobility.
Figure 3: Continued.
We detected the activation of the JAK/STAT3 pathway by western blot. Our results showed that neutrophils activated the JAK/STAT3 pathway, and blockage of IL-17 could effectively reduce the activation of the JAK/STAT3 pathway (Figures 4(a) and 4(b)). To figure out the function of JAK/STAT3 in LSCC progression, we applied the JAK/STAT3 inhibitor in CCK8 (Figures 4(c) and 4(d)), migration (Figures 4(e) and 4(f)), and invasion assays (Figures 4(g) and 4(h)). Results exhibited that the STAT3 inhibitor effectively blocked neutrophil-induced LSCC proliferation, migration, and invasion (Figures 4(c)–4(h)).

3.5. IL-17/JAK/STAT3 Pathway Contributes to LSCC Progression. We used IL-17 instead of neutrophil conditioned medium to confirm the function of the IL-17/JAK/STAT3 pathway in LSCC progression. CCK8 results showed that IL-17 played a similar role as neutrophil conditioned medium in promoting the proliferation of Tu177 and Tu686 (Figures 5(a) and 5(b)), and inhibition of STAT3 could effectively block the proliferation enhancement induced by of IL-17.

Further study showed that IL-17 also significantly enhanced LSCC migration and invasion, and JAK/STAT3 pathway inhibition effectively reduced the enhancement of mobility (Figures 5(c)–5(f)). What is more, western blot results showed that IL-17 also promoted EMT progression. E-cadherin was significantly downregulated with the addition of IL-17 and upregulated with the addition of the STAT3 inhibitor. Vimentin and Snail were found to increase with the addition of IL-17 and decreased when STAT3 activation was inhibited (Figures 5(g) and 5(h)). EMT-related marker changes might contribute to the enhanced migration and invasion abilities triggered by neutrophils. In summary, the experiments confirmed that the IL-17/JAK/STAT3 pathway was responsible for neutrophil-induced LSCC progression.
Figure 4: Continued.
4. Discussion

LSCC is characterized by metastasis and recurrence, which lead to the poor survival of LSCC patients. The incidence of LSCC has gradually increased, while the development of LSCC treatment has stagnated [5]. LSCC has been a heavy burden for global health. Studying the specific mechanism of LSCC progression is of great value for LSCC diagnosis and treatment.

Immune cells are reported to play important roles in disease progression, especially cancer [29, 30]. Neutrophils account for 50% to 80% of leukocytes, which are critical factors in cancer microenvironment. The ratio of neutrophil to lymphocyte is an independent predictor for LSCC overall survival and progression-free survival [31, 32]. What is more, tumor-infiltrating neutrophils are found to promote LSCC progression [33]. However, how neutrophils infiltrate into tumor tissue and how neutrophils contribute to the
Figure 5: Continued.
LSCC progression still remain unclear. In this study, we tried to figure out the complex interaction between neutrophils and LSCC cancer cells. Our research showed that LSCC cells could promote neutrophil activation and mobility. And, in turn, neutrophils promoted the progression of LSCC. We uncovered the crosstalk between LSCC and neutrophils, which would provide new thoughts on LSCC researches.

The functions of IL-17 in cancer are reported to be controversial [34]. IL-17 has both tumor-promoting and tumor-suppressing functions [35]. IL-17 exerts tumor-promoting effects through enhanced signal transduction, angiogenesis, and tissue remodeling. IL-17 could stimulate tumor proliferation and self-renewal and promote tumor infiltration and angiogenesis by activating downstream transcription factors (STAT, NF-κB, and API), antiapoptotic proteins (mTOR, Akt, Bcl-2, Erk, and Bax), and kinases (MAPK and HER1) [36, 37]. IL-17 also promotes cancer progression by changing the microenvironment of immune cells by cytokines and chemokines [38]. Interestingly, IL-17 could also exert tumor-suppressing properties and correlate with better survival in various cancers such as chronic lymphocytic leukemia and gastric cancer [39, 40].

In our study, we found that neutrophils regulate the progression of LSCC through IL-17 secretion. And IL-17 exerts promoting functions in LSCC proliferation, migration, and invasion. We provided some new thoughts on LSCC target therapy.

STAT3 is a cytoplasmic transcription factor which belongs to the STAT family (signal transducer and activator of transcription family). STAT3 is reported to participate in various biological processes such as proliferation, mobility, and stemness [41, 42]. Hyperactivation of STAT3 is widely confirmed in numerous cancers and related to poor prognosis [43]. What is more, hyperactivation of STAT3 is found to regulate the immune microenvironment of the tumor [44] [45]. The JAK/STAT3 pathway is a potential target for proliferation, metastasis, chemoresistance, and immunity. In our research, we uncovered that IL-17 derived from neutrophils could activate the JAK/STAT3 pathway. And activation of JAK/STAT3 could promote the progression of LSCC. Our researches on the JAK/STAT3 pathway exhibit oncogenic roles in LSCC and might provide some new thoughts on LSCC therapy.

5. Conclusion

In conclusion, our research uncovered that LSCC cancer cells could activate neutrophils and promote the mobility of neutrophils. In return, neutrophils promoted proliferation of LSCC. Further study showed that neutrophils activated JAK/STAT3 in LSCC cells through secreting IL-17. Our research showed the complex crosstalk between neutrophils and LSCC, which would provide more thoughts on LSCC target therapy.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors confirmed that there was no conflict of interest.

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