Downregulation of FOXP3 in neutrophils by IL-8 promotes the progression of oral squamous cell carcinoma

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Abstract. The aim of the present study was to investigate the effects of the transcription factor forkhead box P3 (FOXP3) in neutrophils on the progression of oral squamous cell carcinoma (OSCC). Cancer tissue samples and paracarcinoma tissues were collected from 23 patients with OSCC for the current study. In addition, SCC-9, a human tongue carcinoma cell line, was co-cultured with primary human neutrophils and treated with recombinant interleukin 8 (IL-8). The effect of FOXP3 on the proliferation of SCC-9 cells was analyzed using a Cell Counting Kit 8 assay. FOXP3 protein expression in neutrophils was analyzed by quantitative PCR following IL-8 treatment.

FOXP3 protein expression in neutrophils and the amount of IL-8 protein in the OSCC tumor microenvironment were determined by immunofluorescence analysis. The present study demonstrated that IL-8 downregulated FOXP3 mRNA expression in neutrophils. Neutrophils and peptide P60, a specific inhibitor of FOXP3, increased proliferation of SCC-9 cells. In patients with OSCC, FOXP3 protein expression in neutrophils of the stage IV group was significantly lower compared with that in paracarcinoma tissues. In summary, IL-8 in the tumor microenvironment may recruit neutrophils, and downregulation of FOXP3 in neutrophils by IL-8 may promote the progression of OSCC.

Introduction

In 2012, 300,400 newly diagnosed cases of oral cancer were reported, and 145,400 patients succumbed to oral cancer worldwide (1). Oral squamous cell carcinomas (OSCCs) are the most common type of oral cancer (2). Interleukin (IL)-8 is a key cytokine that promotes tumor progression (3). Additionally, a previous study demonstrated that OSCC cells may secrete IL-8 (4). IL-8 released by tumor cells recruits neutrophils from the circulating blood to the local tumor microenvironment (3). Neutrophils entering the tumor microenvironment exert various biological functions, including promotion of tumor angiogenesis and tumor cell proliferation (5,6).

The forkhead/winglike spiral transcription factor forkhead box P3 (FOXP3) is a member of the forkhead transcription factor family (7). As a key transcription factor, FOXP3 serves an important role in the formation of regulatory T cells (Tregs) and their immunosuppressive function (7). Several studies on FOXP3 have focused on Tregs (8-10), and previous studies demonstrated that FOXP3 is expressed not only in Tregs, but also in various types of cancer (11 -13), including pancreatic cancer (11). A study by Hinz et al (11) indicated that pancreatic cancer cells expressing FOXP3 attenuate activated T-cell proliferation. By specifically reducing FOXP3 expression in pancreatic cancer cells, its inhibitory effect on T-cell proliferation can be partially reduced (11).

Neutrophil infiltration of a tumor microenvironment is a common manifestation of tumor pathology (5,6,14,15). Tumor-associated neutrophils (TANs) are classified as the N1 type, with an antitumor effect, and the N2 type, with a tumor-promoting effect (16,17). The phenotype of TANs is associated with factors in the tumor microenvironment, including transforming growth factor β and interferon β (16,17). A study by Nozawa et al (18) revealed that TANs promote tumor cell proliferation in pancreatic cancer. Additionally, neutrophils are not only common immune killer cells, but also a potential immunoregulatory cell type (19). Whether FOXP3 is expressed in neutrophils and whether its expression serves a role in tumor progression, to the best of our knowledge, has not been reported on so far.

In the present study, quantitative PCR (qPCR) was performed to detect the effect of cytokine IL-8 on the expression levels of FOXP3 in neutrophils, and a Cell Counting Kit 8...
(CCK-8) proliferation assay was used to evaluate the effect of FOXP3 expression on the proliferation of OSCC cells in vitro. Furthermore, immunofluorescence staining was conducted to detect the expression levels of FOXP3 in neutrophils in OSCC tissue samples in vivo. The present study broadens the range of known mechanisms via which neutrophils promote tumor cell proliferation and tumor progression.

Materials and methods

Human samples. Cancer tissue samples and paracarcinoma tissues (1.5 cm away from the cancer) were collected from patients with OSCC at the College of Stomatology, Guangxi Medical University (Nanning, China) between July 2017 and December 2017. Patients with OSCC included in the present study had not been previously treated, and were candidates for surgical resection of primary tumors and selective or radical neck dissection. The exclusion criteria were: i) Patients with other severe systemic disorders or distant metastasis; and ii) patients with samples that were inadequate for immunofluorescence staining.

Samples from 23 patients with OSCC were included in the present study. The median age of the patients was 52 years (range, 24-82 years); 14 patients were male, and 9 patients were female. According to the Union for International Cancer Control 7th Edition staging system (20), the patients were divided into stage II (n=6), III (n=7) and IV (n=10) groups. Each sample was stained using hematoxylin and eosin as described previously (21). Histopathological diagnoses were confirmed by at least two independent pathologists. Notable intercellular bridging and keratinized beads were observed in the cancer tissues, histological features which are consistent with squamous cell carcinoma. Tissue samples were soaked in 10% buffered formalin at room temperature for 48 h, followed by paraffin embedding. Blood samples were obtained from 9 healthy donors (5 men and 4 women; median age, 45; age range, 28-56) and placed into heparinized tubes at room temperature and processed within 2 h of blood collection. The Ethics Committee of Guangxi Medical University approved the study protocol, and all patients and healthy donors provided written informed consent.

Cells and culture. SCC-9 cells were obtained from the Fuheng Cell Center (Shanghai, China). The cell line was authenticated by Shanghai Biowing Applied Biotechnology Co., Ltd., using short tandem repeat (STR) profiling (22), according to the American National Standards Institute Standard (ASN-0002) set forth by the American Type Culture Collection Standards Development Organization (22). The STR results revealed that this cell line had no multiple alleles and no cross contamination of human cells. The DNA of the cell line was found to match perfectly with the type of cells in a cell line retrieval, and the DSMZ database (dszmd.de/services/human-and-animal-cell-lines/online-str-analysis) demonstrated that the cells, called SCC-9, corresponded to the cell number CRL-1629. SCC-9 cells were maintained in DMEM/F12 medium (cat. no. 319-085-CL; Wisent Biotechnology) supplemented with 10% FBS (cat. no. 086-110; Wisent Biotechnology) and 1% penicillin/streptomycin solution (cat. no. 450-201-EL; Wisent Biotechnology). Neutrophils were isolated from healthy donor blood samples by means of Polymorphprep™ (cat. no. 1114683; Axis-Shield Diagnostics, Ltd.) according to the manufacturer's protocol, and neutrophils were resuspended in RPMI-1640 medium (cat. no. 350-006-CL; Wisent Biotechnology) supplemented with 10% FBS and 1% penicillin/streptomycin solution. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2, and all subsequent culturing were performed under the same conditions.

Reverse-transcription (RT)-qPCR. To examine the effects of IL-8 on FOXP3 expression in neutrophils, neutrophils (1x10⁶ cells/well) were cultured in 24-well plates in RPMI-1640 medium supplemented with 10% FBS and treated with recombinant human IL-8 (100 ng/ml; cat. no. 200-08; PeproTech, Inc.) or peptide P60 (P60; 100 µM; cat. no. 350582; Abbiotec, Inc.), a specific peptide inhibitor of FOXP3 (24). The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO2.

Ilization of neutrophils and co-culture with SCC-9 cells. To isolate neutrophils, heparinized blood was layered on Polymorphprep according to the manufacturer's protocol. Briefly, 5 ml heparinized blood was carefully layered over 5 ml Polymorphprep in a 15 ml centrifuge tube. The samples were centrifuged at 500 x g for 30 min at 20°C. Subsequently, the lower phase containing neutrophils was collected, diluted with PBS (cat. no. P1010; Beijing Solarbio Science & Technology Co., Ltd.) and centrifuged at 400 x g for 10 min at 20°C. The neutrophils were resuspended in RPMI-1640 medium supplemented with 10% FBS.

The co-culture experiments were conducted in 96-well plates, as described previously (23). The SCC-9 cells were plated at a density of 5x10³ cells/ml in DMEM/F12 medium containing 10% FBS. After 24 h, the medium was discarded, and the cells were incubated in 100 µl RPMI-1640 medium supplemented with 10% FBS. Neutrophils were directly added to the tumor cells at a final density of 5x10⁵ cells/ml. To investigate the effect of FOXP3 on the proliferation of SCC-9 cells in co-culture, the cells were treated with IL-8 (100 ng/ml; cat. no. 200-08; PeproTech, Inc.) or peptide P60 (P60; 100 µM; cat. no. 350582; Abbiotec, Inc.), a specific peptide inhibitor of FOXP3 (24). The plates were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO2.

Cell proliferation assay. Cell proliferation was assessed using the CCK-8 assay (cat. no. 70-CKK805; Hangzhou MultiSciences (Lianke) Biotech Co., Ltd.) according to the manufacturer's protocol. For co-culture experiments, SCC-9 cells were plated at a density of 5x10³ cells/ml in DMEM/F12 medium containing 10% FBS. After 24 h, the medium was discarded, and the cells were incubated in 100 µl of RPMI-1640 medium supplemented with 10% FBS. Neutrophils were directly added to the tumor cells at a final density of 5x10⁵ cells/ml. To investigate the effect of FOXP3 on the proliferation of SCC-9 cells in co-culture, the cells were treated with recombinant IL-8 (100 ng/ml; cat. no. 200-08; PeproTech, Inc.) or P60. Subsequently, 100 µl RPMI-1640 medium containing 10% FBS and 10 µl CCK-8 reagent was added. Optical density was measured at 450 nm on a microplate reader after 2 h. The experiment was independently repeated three times.

Expression of FOXP3 in neutrophils (CCK-8) proliferation assay was used to evaluate the effect of FOXP3 expression in neutrophils, neutrophils (1x10⁶ cells/well) were cultured in 24-well plates in RPMI-1640 medium supplemented with 10% FBS and treated with recombinant human IL-8 (100 ng/ml, diluted in distilled water) or treated with the same volume of PBS (control group) for 12 h. For mRNA analysis, RNA (200 ng per sample) was extracted from neutrophils using RNAiso Plus reagent (cat. no. 9108; Takara Bio, Inc.) according to the manufacturer's
protocol. Complementary DNA (cDNA) was synthesized with the PrimeScript™ RT Reagent kit with gDNA Eraser (Perfect Real Time) (cat. no. RR047A; Takara Bio, Inc.) according to the manufacturer’s protocol. The reverse transcription temperature protocol used was: 37˚C for 15 min followed by 85°C for 5 sec. The primers for GAPDH and FOXP3 were purchased from Takara (Takara Bio, Inc.). The primer sequences were as follows: FOXP3 forward, 5'-GGA ACAGCACATCCCGAGTTC-3’ and reverse, 5'-ATGGCC CAGCAGTGGAG-3’ (25); and GAPDH forward, 5'-GCA CCGTCA AGGCTGAGAAC-3’ and reverse, 5'-TGTTGA AGACGGCGATGGA-3’. GAPDH was used as an internal control. qPCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using TB Premix Ex Taq™ II (cat. no. RR820A; Takara Bio, Inc.) according to the manufacturer's protocol. The thermocycling conditions were: Denaturation at 95°C for 30 sec; followed by 40 cycles at 95°C for 5 sec and 60°C for 34 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. The experiment was independently repeated three times. The 2^\Delta\Delta C_q method was used to calculate the fold in gene expression determined from quantitative PCR experiments (26,27). The fold change in cDNA of the target gene relative to the GAPDH endogenous control was determined by the following equation: Fold change=2^-\Delta\Delta C_q, where \Delta\Delta C_q=(C_q^{FOXP3}−C_q^{GAPDH}) (experimental group)−(C_q^{GAPDH}−C_q^{GAPDH}) (control group). The Ct value is the number of amplification cycles at which the fluorescence signal reaches a set threshold.

**Immunofluorescence staining.** Tissue samples were cut into 4-μm-thick sections. The sections were deparaffinized with xylene and rehydrated in a descending ethanol series of 70, 80, 90, 95 and 100%, followed by antigen retrieval with citrate buffer (cat. no. mvs-0066; Fuzhou Maixin Biotech Co., Ltd.) with pH 6.0, microwaved on high power to boiling point for 3 min and subsequently blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. SW3015) for 30 min. The sections were incubated at 4°C overnight with the following primary antibodies at 1:100 dilution: Rabbit polyclonal anti-human FOX3 antibody (cat. no. ab10901; Abcam), mouse monoclonal anti-human myeloperoxidase (MPO) antibody (cat. no. ab25989; Abcam) and mouse monoclonal anti-human IL-8 antibody (cat. no. sc-8427; Santa Cruz Biotechnology, Inc.). The sections were washed with PBS, followed by incubation with the following secondary antibodies at 1:1,000 dilution for 1 h at 37°C: Goat anti-rabbit immunoglobulin G (IgG) antibody (cat. no. ab150077; Abcam) and goat anti-mouse IgG antibody (cat. no. ab150114; Abcam). Sections were washed with PBS followed by incubation with DAPI (5 μg/ml; cat. no. C0060; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C for 10 min. The tissue sections were washed with PBS before mounting on the slides with Solarbio Fluorescence Mounting medium (cat. no. S2100; Beijing Solarbio Science & Technology Co., Ltd.). SCC-9 cells were fixed with 4% paraformaldehyde at room temperature, and permeabilized with 0.3% Triton X-100 (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. T8200) for 20 min. After blocking with 5% BSA at 37°C for 30 min, the samples were stained as described above. Images were captured under a fluorescence microscope (magnification, x400; Olympus Corporation), and the integrated optical density (IOD) of FOXP3 protein expression in three randomly selected fields was measured using Image Pro-Plus 6.0 software (version 6.0; Media Cybernetics, Inc.). The IOD mean value of each section was determined after analyzing three random images.

**Statistical analysis.** Each experiment was repeated three times and the data are expressed as the mean ± standard deviation. IBM SPSS Statistics software (version 20.0; IBM Corp.) was used to perform statistical analyses. A paired Student’s t-test was used to analyze the difference between FOXP3 mRNA expression in neutrophils. An unpaired Student’s t-test was used to analyze the immunofluorescence results of FOXP3 protein expression in neutrophils. Cell proliferation assays were analyzed using a one-way ANOVA with a post-hoc Tukey’s. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Neutrophils and IL-8 promote SCC-9 cell proliferation.** OSCC cells secrete IL-8, and high concentrations of IL-8 recruit neutrophils to the cancer microenvironment; thus, there are large quantities of IL-8 and neutrophils in the tumor microenvironment (3,4). To investigate the effects of IL-8 and neutrophils on the proliferation of OSCC cells, neutrophils and OSCC cells were co-cultured and treated with IL-8 for 24 h, and SCC-9 cell proliferation was assessed using a CCK-8 assay. The results revealed that IL-8 and neutrophils exerted a synergistic effect on SCC-9 cells and together promoted their proliferation (P<0.001; Fig. 1).

It has previously been shown that neutrophils adhere to the surface of epithelial cells through adhesion molecules (28), and IL-8 promotes the adhesion of neutrophils by upregulating the expression of these adhesion molecules (29). To confirm that the increased proliferation in the SCC-9/neutrophil/IL-8 group was not due to increased adhesion promoted by neutrophils, after washing, neutrophils were detected by immunofluorescence staining using an MPO antibody, which has been used as a neutrophil-specific antibody in previous studies (30,31). The results confirmed that there were no adherent neutrophils on the surface of SCC-9 cells in 96-well plates after washing (Fig. S1).

**IL-8 downregulates FOXP3 mRNA expression in neutrophils.** Various studies have demonstrated that FOXP3 is expressed in a variety of tumor cells and is involved in tumor cell proliferation and apoptosis (11,12,32). To test if IL-8 induces a change in FOXP3 expression in neutrophils, human peripheral blood neutrophils were stimulated with recombinant human IL-8 and the mRNA expression levels of FOXP3 in neutrophils were evaluated. The results indicated that IL-8 downregulated FOXP3 mRNA expression in neutrophils (P=0.005; Fig. 2).

**Neutrophils and an inhibitor of FOXP3 promote proliferation of SCC-9 cells.** To investigate the effect of FOXP3 in neutrophils on the proliferation of SCC-9 cells, neutrophils and OSCC cells were co-cultured and treated with P60, a specific peptide inhibitor of FOXP3, for 24 h. Subsequently, SCC-9 cell proliferation was assessed using a CCK-8 assay. The
results revealed that P60 treatment of co-cultured neutrophils and SCC-9 cells increased the proliferation of SCC-9 cells in comparison with SCC-9 cells alone (P=0.004; Fig. 3), suggesting that a combination of neutrophils and an inhibitor of FOXP3 together promote the proliferation of SCC-9 cells.

Protein expression levels of FOXP3 in neutrophils in the OSCC tumor microenvironment. To investigate the association of FOXP3 in neutrophils and OSCC in vivo, cancer tissue samples from 23 patients were stained by immunofluorescence to detect FOXP3 expression with small interfering RNAs in pancreatic cancer cells. This result indicates that pancreatic cancer cells expressing FOXP3 inhibit T-cell proliferation, and thereby promote tumor progression. However, FOXP3 also performs a tumor suppressor function in breast cancer cells (37,38). Zhang et al (37) reported that FOXP3 is expressed in breast cancer cells and is negatively associated with breast cancer metastasis. Furthermore, a previous study demonstrated that FOXP3 inhibits adhesion and invasiveness of breast cancer cells by downregulating CD44 (37). To date, however, FOXP3 expression and its role in neutrophils, to the best of our knowledge, have not been reported on. The present study demonstrated that IL-8 protein expression is also altered in vivo, immunofluorescence was conducted to detect IL-8 protein expression in cancer and paracarcinoma tissue samples from patients with OSCC. The results demonstrated that IL-8 protein was expressed in both OSCC tissues and paracarcinoma tissues (Fig. 5).

Discussion

Recent studies (34-36) suggest that FOXP3 is expressed in a variety of tumor cells and its protumor or antitumor roles are a controversial topic. Hinz et al (11) demonstrated that T-cell proliferation is observed after specific silencing of FOXP3 expression with small interfering RNAs in pancreatic cancer cells. This result indicates that pancreatic cancer cells expressing FOXP3 inhibit T-cell proliferation, and thereby promote tumor progression. However, FOXP3 also performs a tumor suppressor function in breast cancer cells (37,38). Zhang et al (37) reported that FOXP3 is expressed in breast cancer cells and is negatively associated with breast cancer metastasis. Furthermore, a previous study demonstrated that FOXP3 inhibits adhesion and invasiveness of breast cancer cells by downregulating CD44 (37). To date, however, FOXP3 expression and its role in neutrophils, to the best of our knowledge, have not been reported on. The present study demonstrated that IL-8 downregulated the expression of FOXP3 in neutrophils, and following P60 inhibition of FOXP3, neutrophils promoted the proliferation of SCC-9 cells. A study by Casares et al (24) reported that P60 enters the cells, downregulates FOXP3 nuclear translocation and inhibits the function of FOXP3 protein in vitro. It has been identified that P60 alone
Figure 4. FOXP3 protein expression in neutrophils in the oral squamous cell carcinoma tumor microenvironment. (A) FOXP3 (green) and MPO (red) expression in 23 cancer tissue samples at stage II, III and IV was detected by immunofluorescence. Nuclei (blue) were counterstained with DAPI. Scale bar, 20 µm. (B) FOXP3 expression was quantified based on IOD of fluorescence staining. Data are presented as the mean ± SD. **P<0.01. FOXP3, forkhead box P3; MPO, myeloperoxidase; IOD, integrated optical density.

Figure 5. IL-8 protein expression in oral squamous cell carcinoma and paracarcinoma tissue samples. IL-8 protein expression (red) was detected by immunofluorescence. Nuclei (blue) were counterstained with DAPI. Scale bar, 20 µm. IL-8, interleukin 8.
does not alter effector T cell or Treg proliferation in response to stimulation in vitro (24). In the present study, SCC-9 cells were treated with P60 for 24 h, and there was no statistically significant difference identified in the proliferation of SCC-9 cells compared with the control group (P=0.656). Therefore, P60 alone may not affect the proliferation of SCC-9 cells. The results revealed that IL-8 downregulated FOXP3 expression in neutrophils, but its specific signaling pathway was not examined in the present study. According to other studies, IL-6 and IL-27 inhibit FOXP3 expression by activating the STAT3 signaling pathway (39,40). Furthermore, a study by Qu et al (41) revealed that IL-8 increased STAT3 phosphorylation, whereas knockout of IL-8 reduced phosphorylation of STAT3, suggesting that IL-8 may also suppress FOXP3 expression in neutrophils by activating the STAT3 signaling pathway.

TANs serve an important role in the progression of tumor development. For example, neutrophil-released neutrophil elastase (NE) causes a release of growth factors, thereby promoting tumor cell proliferation (6). NE also promotes tumor cell proliferation by degrading insulin receptor substrate 1 (42). In addition, in the tumor microenvironment, neutrophils promote the proliferation of tumor cells by releasing neutrophil extracellular traps (NETs) into the microenvironment (43,44). The present study revealed that IL-8 downregulated FOXP3 expression in neutrophils, and IL-8 treatment combined with co-culturing with neutrophils promoted the proliferation of SCC-9 cells. In addition, the present study demonstrated that the expression of FOXP3 in neutrophils in samples from patients with stage IV tumors was lower compared with that in stage III and II patients. This suggested that downregulation of FOXP3 in neutrophils in the cancer microenvironment may be associated with progression of OSCC. Chung et al (45) demonstrated that in Foxp3-deficient mice, the microglia produced increased quantities of reactive oxygen species (ROS) when treated with lipopolysaccharide compared with the wild-type mice. FOXP3 negatively regulated the production of ROS by activating NF-κB (45). A previous study on NETs suggested that increased levels of ROS in neutrophils can promote the formation and release of NETs (46). Considering the results of these previous studies, it was hypothesized that IL-8 recruited neutrophils from the blood vessel to the local tumor microenvironment and downregulated FOXP3 expression in neutrophils. The downregulation of FOXP3 expression in neutrophils may subsequently lead to increased production of ROS in neutrophils, and potentially promote the production and release of NETs, stimulating the proliferation of OSCC cells.

In summary, IL-8 in the tumor microenvironment may recruit neutrophils, and downregulation of FOXP3 in neutrophils by IL-8 may promote the progression of OSCC. These findings expand the range of known mechanisms through which neutrophils promote proliferation of tumor cells and thus, tumor progression. However, additional studies are required to fully elucidate the underlying mechanisms.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

FL and HK conceived the study and participated in its design. CZ and FL drafted the manuscript. CZ, XC and WF performed the cell experiments. QT, ZZ and TY performed sample collection and immunofluorescence staining. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Guangxi Medical University. Written informed consent was provided by all patients and healthy volunteers.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


