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MicroRNA-155 regulates cervical cancer via inducing Th17/Treg imbalance

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Abstract. – OBJECTIVE: To explore the effect of microRNA-155 on cervical cancer and its underlying mechanism.

PATIENTS AND METHODS: Peripheral blood and cervical cancer tissues were collected. We used quantitative Real-time polymerase chain reaction (qRT-PCR) to detect expressions of microRNA-155, SOCS1, Th17-related genes (RORγt, IL-17A, IL-21, and IL-22), and Treg-related genes (foxp3, TGF-β, IL-10, and IL-35) in peripheral blood and cervical cancer tissues. Western blot was used to detect protein expressions of RORγt and foxp3. The proportions of Th17 and Treg cells in CD4+ T cells were measured by flow cytometry. Moreover, IL-17 expression was detected by enzyme-linked immunosorbent assay (ELISA).

RESULTS: MicroRNA-155 was overexpressed in peripheral blood and cervical cancer tissues of patients with cervical cancer compared with those of normal controls. Th17-related transcription factors and cytokines in cervical cancer tissues were remarkably elevated than those of normal controls, including RORγt, IL-17, and IL-6. Treg-related transcription factors and cytokines obtained the similar results. Besides, the proportion of Th17 cells in CD4+ T cells was higher in cervical tissues than that of normal controls. *In vitro* experiments suggested that overexpressed microRNA-155 can inhibit the expression of target gene SOCS1, promote the differentiation of Th17 and increase levels of IL-17, RORγt, and STAT3.

CONCLUSIONS: MicroRNA-155 is involved in the occurrence and progression of cervical cancer via inhibiting SOSC1 expression and inducing Th17/Treg imbalance.

Key Words

Cervical cancer, MicroRNA-155, Immune regulation, Th17/Treg.

Introduction

Cervical cancer is a common malignant tumor of the reproductive system in women. The prevalence and mortality rates of cervical cancer are the fourth and third in female malignancies, respectively. More seriously, 80% of patients with cervical cancer are from developing countries[1]. In recent years, the incidence of cervical cancer has been rapidly risen, which presents a shorter disease course and younger onset. Although screening for cervical cancer has been widely applied, a great number of patients are still diagnosed as invasive cervical cancer[2]. So far, the 5-year survival rate of patients with cervical cancer after standard treatment is still low[3]. It is urgent to further explore the underlying mechanism, thus providing new directions for individualized treatment of patients with cervical cancer.

Accumulating studies have confirmed that the persistent infection of Human Papilloma Virus (HPV) is considered to be the most important pathogenic factor for cervical cancer, which is the initial step in the development of cervical cancer[4]. Multiple microRNAs are differentially expressed in HPV-induced cervical cancer[5,6]. MicroRNAs in humans and other mammalians could regulate at least one-third of the genes in the body with tissue and disease specificities[7-9]. It is suggested that microRNA may participate in the development and progression of cervical cancer.

MicroRNA is a single-stranded, non-coding small molecule RNA, which is endogenously expressed in cells with 19-25 nt in length. Functionally, microRNA induces mRNA degradation or inhibits transcription of target genes, thereby negatively regulating gene expressions[10-12]. MicroRNA-155 is involved in a variety of cellular activities and plays a crucial role in many diseases. Studies have confirmed that microRNA-155 is upregulated in cancers such as cervical cancer[13] and breast cancer[14], and is closely related to the development, progression, and prognosis of tumors. Meanwhile, microRNA-155 is also involved in immune response[15,16] and autoimmune diseases, such as cardiovascular diseases, etc.[17]. However, the specific effect of microRNA-155 on cervical cancer still needs to be elucidated.

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Patients and Methods

Sample Collection

10 cases of cervical cancer and 10 cases of normal controls from October 2015 to October 2017 were enrolled. Peripheral blood and cervical tissue samples from the two groups were harvested, followed by preservation in sterile and enzyme-free cryovials. Cervical cancer samples were pathologically confirmed and patients with cervical cancer did not receive preoperative anti-tumor treatments, such as chemotherapy and radiotherapy. No significant differences were found in the age, tumor size, tumor location, and pathological type between the two groups. The study was approved by the People's Hospital of Weifang Ethics Committee, and all subjects signed the informed consent.

Cell Culture and Transfection

$CD4^+$ T cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibico, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in a 5% CO_2 incubator at 37°C. Cell transfection was performed when cell confluence was up to 70%-80% according to the instructions of Lipofectamine™2000 (Invitrogen Carlsbad, CA, USA). The primers used were as follows: MicroRNA-155 mimics (sense: 5′-UAAUGCUAAUCGUGAUAGGGGU-3′, anti-sense: 5′-AAUUACGAUUAGCACUAUCCCCA-3′); microRNA-155 inhibitor (sense: 5′-AAAACAUUCAUUGUUGUCGGUGG-3′, anti-sense: 5′-CAGUACUUUUGUGUAGUACAA-3′), negative control (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, anti-sense: 5′-ACGUGACACGUUCGGAGAATT-3′). All reagents were purchased from GenePharma (Shanghai, China).

Separation of PBMCs (Peripheral Blood Mononuclear Cells) and Cell Sorting of $CD4^+$ T

6 mL of peripheral blood was collected and then preserved in anticoagulant tubes containing heparin. Isodose phosphate-buffered saline (PBS) was added at room temperature and gently mixed. Then, 6 mL of the lymphocyte separation solution was preserved in a 50 mL centrifuge tube, followed by slow addition of diluted blood at 45° along the wall of the tube. After centrifugation, the cloud layer was harvested into a new centrifuge tube for preparing PBMC suspension with RPMI-1640 medium. Cell number was adjusted to 1×10^7/mL for the following experiments.

For cell sorting, 10 μL of Biotin-Antibody Cocktail was added into 1 mL of PBMC suspension and incubated at 4°C in the dark for 5 min. 20 μL of Anti-Biotin MicroBeads was further added and incubated at 4°C in the dark for 10 min. $CD4^+$ T cell suspension with high purity was obtained by sorting with a midi sorter. All the reagents and industries for cell sorting were purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

We used TRIzol kit (Yeasen, Shanghai, China) to extract total RNA of the hFOB1.19 cells. The extracted mRNA was reversely transcribed to complementary Deoxyribose Nucleic Acid (cDNA) according to the instructions of First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Primers used in this study were showed in Table I.

Western Blot

The total protein was extracted by the radioimmunoprecipitation assay (RIPA) lysate (Yeasen, Shanghai, China). The concentration of each protein sample was determined by bicinchoninic acid (BCA) kit (Abcam, Cambridge, MA, USA). Briefly, total protein was separated by a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under denaturing conditions and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of specific primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight. After washing with Tris-buffered saline-Tween (TBST, Yeasen, Shanghai, China) for 3 times, membranes were incubated with the secondary antibody (Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence method, and the relative protein expression levels were reflected by target protein/reference GAPDH (gray value).

Detection of Treg and Th17 Cells

FITC-CD4 and APC-CD25 were added in 2×10^5 $CD4^+$ T cells and incubated for 30 min at room temperature in the dark. Cells were centrifuged, fixed and permed according to the instructions of Fix&Perm kit (BioLegend, San Diego, CA, USA). After Foxp3 was used for blockage, PE-Foxp3, PE-IL-17, and isotype control antibodies were added, followed by incuba-

Table I. qRT-PCR primer pairs.

Name	Forward	Reverse
RORγt	5'-GCAGGAGCAATGGAAGTCG-3'	5'-CGCTGAGGAAGTGGGAAAA-3'
IL-7A	5'-GCTGTTGCTGCTGCTGAG-3'	5'-TGGAACGGTTGAGGTAGTC-3'
IL-6	5'-ACAACCACGGCCTTCCCTACT-3'	5'-CTCATTTCCACGATTTCCCAGA-3'
IL-21	5'-GGACCCTTGTCTGTCTGGTAG-3'	5'-TGTGGAGCTGATAGAAGTTCAGG-3'
IL-22	5'-ATGAGTTTTCCCTTATGGGGAC-3'	5'-GCTGGAAGTTGGACACCTCAA-3'
Foxp3	5'-GAGAAAGCGGATACCAAA-3'	5'-TGTGAGGACTACCGAGCC-3'
TGF-β	5'-CAAACTAAGGCTCGCCAGTCC-3'	5'-TTGCGGTCCACCATTAGCAC-3'
IL-10	5'-GGACAACATACTGCTAACCGACTC-3'	5'-TTCATGGCCTTGTAGACACCTT-3'
IL-35	5'-TATGGTCAGCGTTCCAACAGC-3'	5'-TTCGGGACTGGCTAAGACACC-3'
STAT3	5'-CTGGTGAACTACTCAGGGTGT-3'	5'-GTTGGTCGCATCCATGATCTTA-3'
SOCS1	5'-TTCCGCTCCCACTCCGATTA-3'	5'-GTCCCCAATAGAAGCCGCAG-3'
β-actin	5'-GTGACGTTGACATCCGTAAAGA-3'	5'-GTAACAGTCCGCCTAGAACAC-3'
microRNA-155	5'-GTCGTATCCAGTGCAGGGTCCGAGGT-3'	5'-CCAGTGCAGGGTCCGAGGTATT-3'
U6	5'-GCTTCGGCAGCACATATACTAAAAT-3'	5'-CGCTTCAGAATTTGCGTGTCAT-3'

tion at room temperature for 2-3 h without light. Finally, cells were fixed by 1% paraformaldehyde for determination using flow cytometry (Partec AG, Arlesheim, Switzerland).

Enzyme-Linked Immunosorbent Assay (ELISA)

$CD4^+$ T cell supernatants were collected and centrifuged, followed by detection of IL-17A level in cells by ELISA assay. The specific procedure was strictly performed according to the ELISA instructions (Abcam, Cambridge, MA, USA). Briefly, after the 96-well plate was pre-coated, the standard sample and antibodies were added. Biotin-labeled horseradish peroxidase (Avidin-HRP) was further applied for developing color. Optical density (OD) value at the wavelength of 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

We used Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) for statistical analysis. The *t*-test was used for the comparison between two groups. $p<0.05$ was considered statistically significant (*$p<0.05$, **$p<0.01$, and ***$p<0.001$).

Results

Upregulated MicroRNA-155 in Cervical Cancer Tissues

MicroRNA-155 was upregulated in cervical cancer tissues (Figure 1A) and peripheral blood of patients with cervical cancer (Figure 1B) compared with that of healthy controls. Our data suggested that microRNA-155 may participate in cervical cancer, which was consistent with that of other investigations[13].

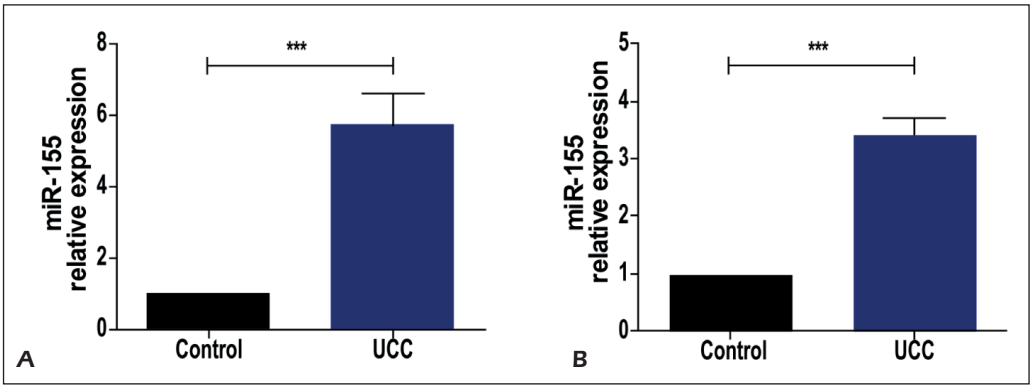

Figure 1. MicroRNA-155 was upregulated in peripheral blood and cervical cancer tissues of patients with cervical cancer. **A**, The expression level of microRNA-155 was upregulated in cervical cancer tissues in comparison with that of normal controls. **B**, The expression level of microRNA-155 was upregulated in peripheral blood of patients with cervical cancer in comparison with that of normal controls.

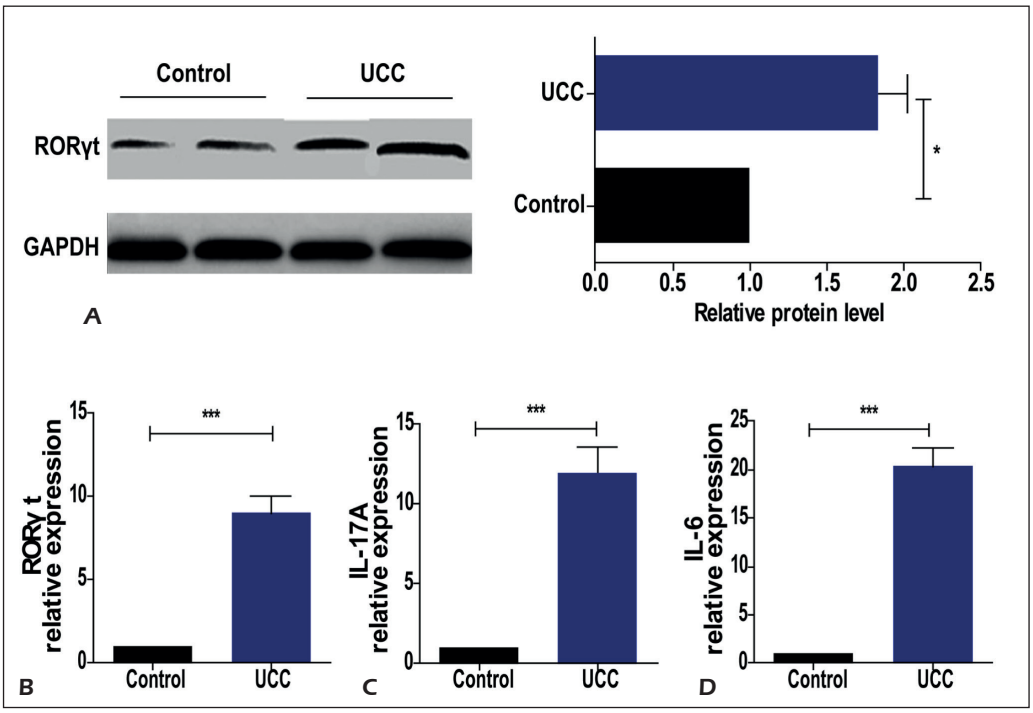

Figure 2. Th17-related factors were upregulated in cervical cancer tissues. **A**, The protein expression level of RORγt was upregulated in cervical cancer tissues in comparison with that of normal controls. **B-D**, The mRNA expression levels of RORγt (**B**), IL-17A (**C**) and IL-6 (**D**) were upregulated in cervical cancer tissues in comparison with those of normal controls, repectively.

Th17-Related Factors Were Over-expressedin Cervical Cancer Tissues

Studies[18,19] have shown that Th17 mainly exerts pro-inflammatory effects by secreting proinflammatory cytokines, including IL-17A, IL-21, and IL-22, which is induced by the transcriptional differentiation of RORγt *via* transforming TGF-β and IL-6. Our study found that the protein (Figure 2A) and mRNA (Figure 2B) expression levels of RORγt in cervical cancer tissues were significantly higher than those in normal controls, respectively. Besides, IL-17A (Figure 2C) and IL-6 (Figure 2D) were also upregulated in cervical cancer tissues in comparison with those of normal controls. The above results indicated that Th17-related factors are upregulated in cervical cancer.

Imbalance of Th17 and Treg Cells in PBMC of Patients With Cervical Cancer

The proportion of Th17 in $CD4^+$ T cells from PBMC of patients with cervical cancer was remarkably increased than that of the control group (Figure 3A), whereas the proportion of Treg cells in both groups was not significantly different (Figure 3B). Treg cells are capable of secreting anti-inflammatory cytokines such as IL-10, IL-35, and TGF-β, which participate in the maintenance of immune tolerance and homeostasis[19,20]. RORγt and foxp3 are key transcription factors of Th17 and Treg cells, respectively. Western blot results showed higher levels of RORγt and foxp3 were found in PBMC of patients with cervical cancer in comparison with those of normal controls (Figure 3C). Moreover, higher mRNA levels of Th17-related genes (Figure 3D) and Treg-related genes (Figure 3E) were observed in PBMC of patients with cervical cancer than those of normal controls. These results indicated that the balance of Th17 and Treg cells is impaired in cervical cancer.

MicroRNA-155 Promoted Differentiation of Th17 Cells

For *in vitro* experiments, we first verified the transfection efficacy of microRNA-155 mimic and microRNA-155 inhibitor, respectively (Figure 4A). SOCS1 serves as a signal regulator of many cytokines, is mainly involved in cell differentiation and tumor development[21]. In this work, SOCS1 was downregulated after overexpression of microRNA-155 (Figure 4B). Overexpressed microRNA-155 also led to the elevated proportion of Th17 cells in $CD4^+$ T cells (Figure 4C).

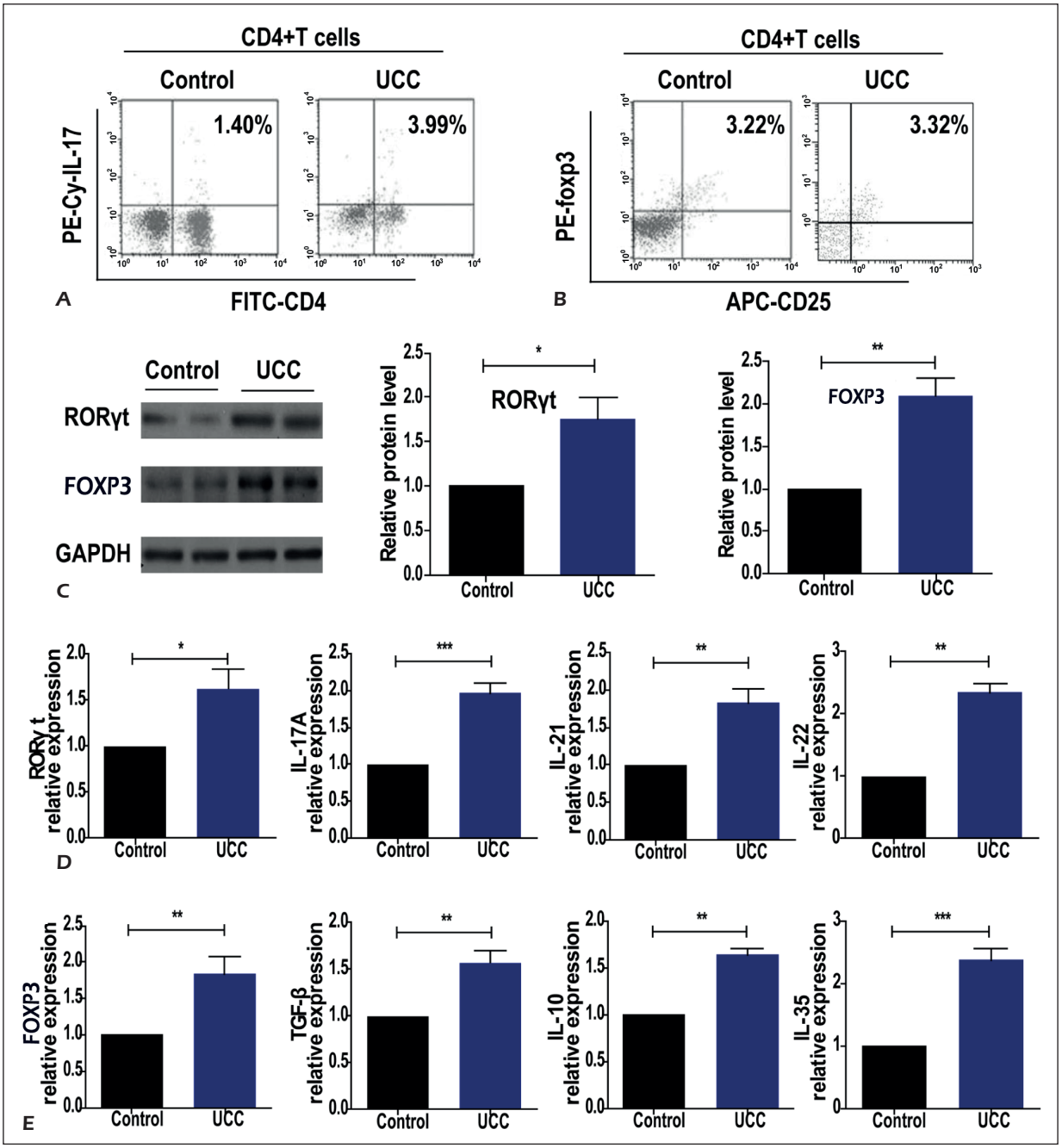

Figure 3. Imbalance of Th17/Treg in peripheral blood of patients with cervical cancer. **A**, The proportion of Th17 in CD4+T cells from PBMCs (Peripheral Blood Mononuclear Cells) of patients with cervical cancer was remarkably increased in comparison with that of normal controls. **B**, No significant difference in the proportion of Treg in CD4+T cells was found between patients with cervical cancer and normal controls. **C**, The protein expression levels of RORγt and FOXP3 in peripheral blood were increased in patients with cervical cancer in comparison with those of normal controls, respectively. **D**, The mRNA expression levels of RORγt, IL-17A, IL-21, and IL-22 were increased in patients with cervical cancer in comparison with those of normal controls, respectively. **E**, The mRNA expression levels of foxp3, TGF-β, IL-10, and IL-35 were increased in patients with cervical cancer in comparison with those of normal controls,respectively.

Besides, increased mRNA (Figure 4D) and protein (Figure 4E) expressions of IL-17 were observed after overexpression of microRNA-155. We next evaluated the effect of RORγt, a key transcription factor of Th17 cells. The data demonstrated that RORγt was upregulated when

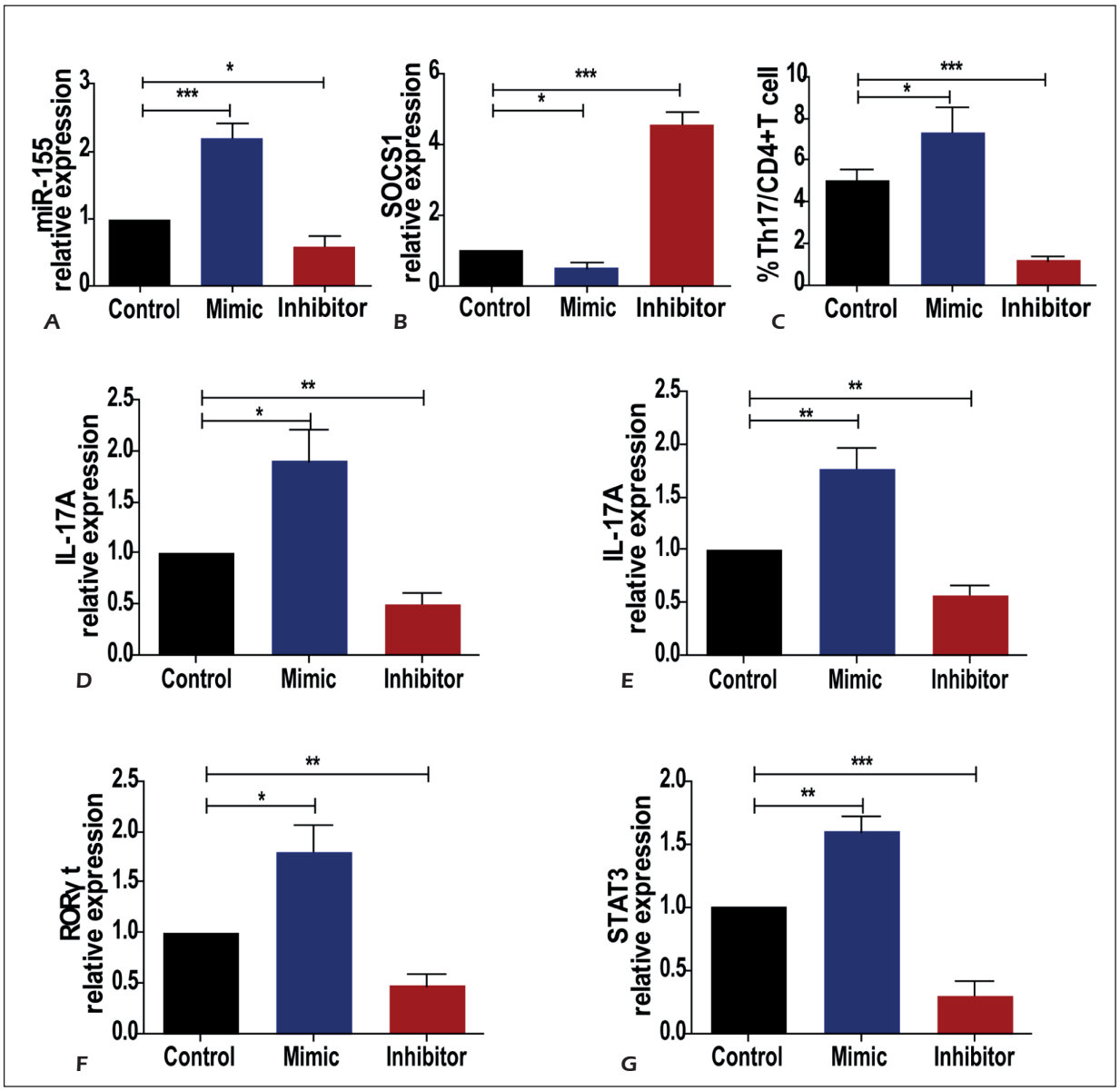

Figure 4. MicroRNA-155 promoted differentiation of Th17 cells. **A**, The mRNA level of microRNA-155. B, The mRNA level of SOCS1. C, Overexpressed microRNA-155 led to the elevated proportion of Th17 cells in CD4+ T cells. **D**-**E**, The mRNA (**D**) and protein (**E**) expressions of IL-17 were increased after overexpression of microRNA-155. **F**, RORγt was upregulated when microRNA-155 was overexpressed. **G**, STAT3 expression was elevated after overexpression of microRNA-155.

microRNA-155 was overexpressed (Figure 4F). It is reported that IL-23/IL-23R/STAT3 pathway participates in the proliferation of Th17 cells and stability maintenance of cell subpopulation[22]. Here we found that STAT3 expression was elevated after overexpression of microRNA-155 (Figure 4G). These results suggested that microRNA-155 can promote the differentiation of Th17 cells.

Discussion

Several researches have shown that the disturbance of the cellular immune response is involved in cervical cancer, which is mainly manifested as immune tolerance. Treg cells are responsible for this immunoregulations, which induce immune tolerance and regulate differentiation of active agents in various immunocompetent cel-

ls, resulting in the decreased immunoregulatory capacity[23]. Th17 cells are a new subpopulation of effector or $CD4^+$ T cells[23,24], which can promote inflammatory responses and strengthen the defense response[25].

Activated $CD4^+$ T cells exert a leading role in tumor immunity, among which, Th17-expressed IL-17 has a significant effect on tumorigenesis[26]. In addition to IL-17, Th17 cells can also express IL-21 and IL-22[27]. These cytokines are beneficial to the pathogens clearance in the body. During the differentiation and development of Th17 cells, secretion of TGF-β, IL-6, IL-23, and IL-21 are mainly regulated by IL-23[28]. Although IL-23 is an important factor in inducing cell effects, it does not affect the differentiation of Th17 cells in the absence of IL-23. It is reported that TGF-β and IL-6 are involved in inducing differentiation, which in turn stimulate Th17-induced secretion of RORγt and IL-17A[29]. TGF-β alone could induce the differentiation of Treg cells, thereby participating in immunosuppression[30]. Intercellular regulation between Th17 and Treg cells are of great significance[31,32].

Treg cells are a group of $CD4^+$ T cells that negatively regulate immunity. Foxp3 is the biomarker of Treg cells, which is also the transcription factor of Treg. Treg cells can strongly inhibit the killing effect of T cell-dominant tumor cells, reduce tumor cells attack and induce immune tolerance of tumors. Functionally, Treg cells exert immunosuppressive effects by direct cell-cell contact, downregulate immune response to autoantigens or alloantigens, and maintain its own immune tolerance. Treg cells could also indirectly exert immunosuppressive effects by secreting IL-10, TGF-β, and other inhibitory cytokines[33].

We found that proportions of Th17 and Treg cells in the cervical tissue and PBMC of patients with cervical cancer were increased. Besides, relative transcription factors were also upregulated compared with those of normal controls, indicating the important effects of Th17 and Treg on the occurrence of cervical cancer[34]. Th17 cells mainly suppress tumor immunity, whereas Treg cells mediate immune escape of tumor cells. Therefore, Th17/Treg imbalance leads to various diseases. Studies have shown that SOCS1 participates in the differentiation of T cells[35]. SOCS1 regulates the differentiation of Th1, Th2, Th17 cells and is associated with a variety of cytokines and signaling pathways[36]. In addition, SOCS1 can inhibit the transformation of Treg into Th17 cells[37]. Our study found upregulated IL-17 and downregulated SOCS1 in $CD4^+$ T cells after microRNA-155 overexpression, indicating that microRNA-155 is crucial in cervical cancer *via* inducing $CD4^+$ T cells differentiation.

Conclusions

We showed that microRNA-155 is involved in the occurrence and progression of cervical cancer *via* inhibiting SOSC1 expression and inducing Th17/Treg imbalance.

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Conflict of Interest

The authors declared no conflict of interest.

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