MiR-203 regulates proliferation and apoptosis of ovarian cancer cells by targeting SOCS3

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Abstract. – OBJECTIVE: Cytokine signal transduction inhibitor 3 (SOCS3) negatively regulates Janus kinases (JAK) - signal transducer and activator of transcription (STAT) pathway. Bioinformatics analysis revealed a targeted relationship between miR-203 and SOCS3 mRNA. This study investigated the role of miR-203 in ovarian cancer cell proliferation and apoptosis.

PATIENTS AND METHODS: Ovarian cancer tissues and adjacent tissues were collected to detect the expression of miR-203 and SOCS3. Ovarian cancer HO8910 cells were divided into miR-NC group, miR-203 inhibitor group, and miR-203 mimic group followed by the analysis of the expression of miR-203 and SOCS3 mRNA by quantitative Reverse Transcription-PCR (qRT-PCR), protein expression of p-JAK2 and p-STAT3 by Western blot, cell apoptosis by flow cytometry, and proliferation by 5-Ethynyl-2'-deoxyuridine (EdU) staining chronologically.

RESULTS: Compared with adjacent tissues, miR-203 expression was significantly increased in tumor tissues and SOCS3 mRNA expression was decreased. Compared with those with lower miR-203 expression, the prognosis of patients with higher expression of miR-203 was significantly worse. There was a targeted regulatory relationship between miR-203 and SOCS3 mRNA. Compared with HO8910 cells, miR-203 expression in HO8910 and SKOV3 cells was increased, and its expressions of SOCS3 mRNA and protein were decreased. Compared with miR-NC group, the transfection of miR-203 inhibitor significantly increased SOCS3 expression, and decreased the expression of p-JAK2 and p-STAT3 protein. We draw the conclusion that miR-203 increased cell apoptosis and decreased cell proliferation. However, opposite results were observed after the transfection of miR-203 mimic.

CONCLUSIONS: Abnormal miR-203 and SOCS3 expression are related to the pathogenesis of ovarian cancer. MiR-203 affects the proliferation of JAK-STAT pathway and regulates the proliferation and apoptosis of ovarian cancer cells by targeting the inhibition of SOCS3 expression.

Key Words: MiR-203, SOCS3, JAK-STAT, Ovarian cancer.

Introduction

Ovarian cancer (OC) is one of the most common malignant tumors of the female reproductive system and has been the 5th leading cause of death in terms of female malignant tumor-related mortality, which poses a serious threat to the health and quality of life of female population. The JAK-STAT signaling pathway is involved in various biological processes, such as cell survival, proliferation, migration, and invasion. SOCS3 is one of the most important and widely expressed member of the cytokine signaling (SOCS) family, which directly inhibits the activity of JAK kinase and phosphorylation of its downstream STAT protein, blocking the activation of the JAK-STAT signaling pathway. Studies have shown that, as a tumor suppressor gene, abnormal expression or functioning of SOCS3 is associated with the occurrence, progression, metastasis, and prognosis of ovarian cancer.

MicroRNA is an endogenous non-coding small-molecule single-stranded RNA of eukaryotes with a length of about 22-25 nucleotides, which binds to the 3'-Untranslated Region (UTR) of the target gene mRNA by complementary pairing to degrade or inhibit translation. MicroRNAs regulate the expression of target genes, which account for 1% of human genes. In ovarian cancer-related studies, several researches have shown that the abnormal expression of miR-203 is closely related to the occurrence, progression, metastasis, and prognosis of ovarian cancer, suggesting that miR-203 plays the role of both tumor
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Patients and Methods

Main Reagents and Materials

Human normal ovarian epithelial cells IOSE80, ovarian cancer HO8910, SKOV3 cells were purchased from Sino Biological (Beijing, China); HEK293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China); Dulbecco's Modified Eagle's Medium (DMEM) and Opti-MEM were purchased from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS) was purchased from Yikesai Biotechnology (Shanghai, China); Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA); PrimeScript™ RT reagent Kit was purchased from TaKaRa (Shanghai, China); SYBR Green dye purchased from Toyobo (Osaka, Japan); miR-203 inhibitor, miR-NC, and miR-203 mimic were designed and synthesized by RiboBio (Guangzhou, China), Rabbit anti-human SOCS3, p-JAK2, p-STAT3 polyclonal antibody were purchased from Abcam (Cambridge, MA, USA); rabbit anti-human β-actin polyclonal antibody was purchased from Boster Bio (Wuhan, China); Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Jackson Immunoresearch (West Grove, PA, USA); Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) apoptosis assay reagent was purchased from Promega (Madison, WI, USA); EdU-Alexa Fluor 488 cell proliferation assay kit, EdU nuclear translocation antibody was purchased from Thermo Fisher Scientific (Waltham, MA, USA); all commonly used related reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Clinical Data

42 patients with ovarian cancer who were treated at The 960th Hospital of the PLA Joint Logistics Support Force from January 2016 to March 2019 were selected, with an average age of 43.6±11.8 years. The tumor tissues that have been removed during the operation and the specimens of the adjacent tissues more than 2 cm away from the tumor tissues were collected, and all the tissue specimens were confirmed by pathological examination. Patients were informed about the collections of the tissues and the procedure was reviewed and approved by The 960th Hospital of the PLA Joint Logistics Support Force Ethics Committee.

Cell Culture

IOSE80, HO8910 and SKOV3 cells were inoculated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin and cultured in a cell culture incubator containing 5% CO₂ at 37°C. When the process was completed, cells were collected by enzymatic digestion, sub-cultured at a ratio of 1:6. The investigation was performed when cells were in a logarithmic growth phase.

Dual-Luciferase Reporter Gene Test

The entire length of the 3'-UTR fragment of the SOCS3 gene or the fragment containing the mutant was amplified using the HEK293T cell genome as a template, and was cloned into the pGL3 vector, transformed into DH5α competent cells, and the correct plasmids were sequenced as 5' AUUCAGAAAAGAAACAUUUCA 3', 3' GAUCACCAGGAUUUGUAAAGU 5' and 5' AUUCAGAAAAGCAGCGCAUGA 3' and named as pGL3-SOCS3-WT, pGL3-SOCS3-MUT. pGL3-SOCS3-WT (or pMIR-SOCS3-MUT) and miR-203 mimic (or miR-NC) were co-transfected into HEK293T cells with Lipofectamine 2000 reagent. After 48 hours of cells culture, the relative Luciferase activity was measured using the Dual-Luciferase Reporter Assay kit.

Cell Transfection

HO8910 cells were cultured in vitro and divided into 3 transfection groups; i.e., miR-NC transfection group, miR-203 inhibitor transfection group, and miR-203 mimic transfection group. The general procedure for transfection was as follows: 10 μL of Lipofectamine 2000 and 50 nmol miR-NC (or miR-203 inhibitor or miR-203 mimic) were diluted with 100 μL of serum-free Opti-MEM; respectively, and then incubated for 5 min at room temperature; respectively, gently mixed and next, incubated for 20 min at room temperature. We added the transfectants to the
QRT-PCR Detection of Gene Expression
Ribonucleic Acid (RNA) was extracted by TRIzol method, and was reversely transcribed to cDNA using PrimeScript™ RT reagent Kit, and the resulting cDNA was stored in a refrigerator at −20°C. Polymerase chain reaction (PCR) amplification was carried out under the action of Taq DNA polymerase using cDNA as a template in a total of 10 μL PCR reaction system (including 2×SYBR Green 5.0 μL, 2.5 μm/L forward primer 0.5 μL, 2.5 μm/L reverse primer 0.5 μL, cDNA 1 μL, and dH2O). PCR conditions: 95°C 5 min, 95°C 15 s, 60°C 1 min, fluorescence data were stored on a Bio-Rad CFX96 real-time PCR instrument (manufacturer: Bio-Rad; model: CFX96, Hercules, CA, USA) for 40 cycles.

Western Blot
The cells were collected, and the total protein was extracted from the radioimmunoprecipitation assay (RIPA) lysate. After the concentration was determined, 40 μg was applied to the sample, and electrophoresis was carried out for 3 h in sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) 12% separation gel, 5% concentrated gel, the protein was electrophorated (300 mA), and then, transferred to polyvinylidene difluoride (PVDF) membrane for 90 min, followed by blocking with 5% skim milk powder in phosphate-buffered saline and Tween 20 (PBST) for 60 min at room temperature, and then incubated with primary antibody at 4°C overnight (the dilution ratios of SOCS3, p-JAK2, p-STAT3, and β-actin are 1:2500, 1:1000, 1:1000, 1:8000, respectively), followed by washing 3 times with PBST, and incubated with HRP-labeled secondary antibody (1:5000 dilution) for 60 min at room temperature, and incubated for 1-3 min at room temperature, followed by the process in terms of exposure and development.

Flow Detection of Cell Apoptosis
The above-mentioned miR-NC, miR-203 inhibitor, and miR-203 mimic transfection group HO8910 cells were collected by trypsinization. After being washed twice with PBS, 300 μL of Binding Buffer was added to the cell pellet and then centrifuged, washed twice with PBS, and then centrifuged again. After the centrifugation, 5 μL of Annexin V-FITC was added, and 5 μL of PI staining solution was added, followed by the analysis of cell apoptosis by flow cytometry.

EdU Staining for Cell Proliferation
HO8910 cells were resuspended in DMEM complete medium containing 10% FBS, and incubated with 10 μM 5-Ethynyl-2'-deoxyuridine (EdU) for 120 min, transferred to the medium according to the procedure described above, and then cultured for 72 h. After the incubation, the cells were fixed according to the procedure. Based on the instructions, after the centrifugation, fixing, permeabilization, and incubation of the reaction solution, cell proliferation was detected by Beckman Coulter FC 500 II flow cytometry (Brea, CA, USA), and the proliferation ability of the cells was reflected by the positive rate of EdU.

Statistical Analysis
Statistical analysis of the data was performed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) 18.0 software. The measurement data were expressed as mean ± standard deviation (SD). The Student’s t-test was used to compare the measurement data between the two groups. p < 0.05 was considered statistically significant.

Results
Abnormal Expression of MiR-203 and SOCS3 in Ovarian Cancer
The results of qRT-PCR showed that the expression of miR-203 was significantly increased in tumor tissues of ovarian cancer patients compared with adjacent tissues (Figure 1A). The results of qRT-PCR showed that the expression of SOCS3 mRNA in tumor tissues of ovarian cancer patients was significantly lower than that of adjacent tissues (Figure 1B).

High Expression of MiR-203 is Associated With Poor Prognosis in Z With Ovarian Cancer
The ovarian cancer patients were divided into miR-203 high expression group and miR-203 low expression group based on the median level of miR-203 mRNA expression. The relationship be-
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Between miR-203 expression and survival rate and prognosis was analyzed. Survival curve analysis showed that the survival rate and prognosis of patients with high miR-203 expression were much worse than those with low expression of miR-203 (Log-rank test $\chi^2 = 4.506, p = 0.034$; Figure 2).

**Targeted Regulation Relationship Between MiR-203 and SOCS3 mRNA**

Bioinformatics analysis revealed a complementary binding site located between miR-203 and the 3'-UTR of SOCS3 mRNA (Figure 3A). Dual-Luciferase gene reporter assays showed that transfection of miR-203 mimic remarkably reduced relative Luciferase activity in pGL3-SOCS3-WT transfected HEK293T cells, but miR-NC or miR-203 mimic did not effect relative Luciferase activity in HEK293T cells transfected with pGL3-SOCS3-MUT (Figure 3B), indicating that miR-203 has a targeted regulatory relationship with the 3'-UTR region of SOCS3 mRNA.

**Figure 1.** Abnormal expression of miR-203 and SOCS3 in ovarian cancer. A, QRT-PCR detection of miR-203 expression in ovarian cancer tissues. B, QRT-PCR detection of SOCS3 mRNA expression in ovarian cancer tissues. *Represents $p < 0.05$ compared with adjacent tissues.

**Figure 2.** High expression of miR-203 is associated with poor prognosis in patients with ovarian cancer.

**Figure 3.** Targeted regulation relationship between miR-203 and SOCS3 mRNA. A, Schematic diagram of the interaction site between miR-203 and the 3'-UTR of SOCS3 mRNA. B, Dual-Luciferase gene reporter assay. *Represents $p < 0.05$ compared to miR-NC.
Increased Expression of miR-203 in Ovarian Cancer Cells and Decreased Expression of SOCS3

The results of qRT-PCR showed that compared with normal ovarian epithelial IOSE80 cells, the expression of miR-203 in ovarian cancer HO8910 and SKOV3 cells was significantly increased, while the expression of SOCS3 mRNA was significantly decreased (Figure 4A). Western blot analysis showed that the expression of SOCS3 protein in ovarian cancer HO8910 and SKOV3 cells was significantly lower than that in normal ovarian epithelial IOSE80 cells (Figure 4B).

Inhibition of miR-203 Expression Attenuates Ovarian Cancer Cell Proliferation and Induces Apoptosis

The results of qRT-PCR showed that compared with miR-NC transfection group, the expression of miR-203 was significantly decreased in HO8910 cells transfected with miR-203 inhibitor, whereas the expression of SOCS3 mRNA was significantly increased. The expression level of miR-203 was significantly increased in the HO8910 cells transfected with miR-203 mimic, while the expression level of SOCS3 mRNA was significantly decreased (Figure 5A). Western blot analysis showed that compared with miR-NC transfection group, transfection of miR-203 inhibitor significantly increased the expression of SOCS3 protein in HO8910 cells and significantly decreased the expression of p-JAK2 and p-STAT3 protein. Transfection of miR-203 mimic reduced the expression of SOCS3 protein and increased the expression of p-JAK2 and p-STAT3 protein (Figure 5B). Flow cytometry results showed that the proliferation of HO8910 cells in the miR-203 inhibitor transfection group was significantly reduced compared with miR-NC transfection group (Figure 5C), and cell apoptosis was significantly increased (Figure 5D). On the other hand, miR-203 mimic transfection significantly increased the proliferation of HO8910 cells and reduced apoptosis.

Discussion

Ovarian cancer is rather insidious in the early stage. Yet it's well known for its rapid progression. Most patients are in the middle or late stages of the disease when they are admitted to hospital for treatment, which somewhat added difficulties to the treatment. Therefore, to explore the pathogenesis of ovarian cancer and to find the signal molecules of abnormal changes have the potential of great significance in helping to diagnose, improve the therapeutic effect, as well as the prognosis of patients.

When a cytokine, i.e., a growth factor or a ligand, binds to an intracellular receptor, the receptor can form a homologous or heterodimer, and phosphorylate the JAK kinase. The activated JAK can make the receptor phosphorylation of the tyrosine residue of STAT to complement STAT via the SH2 domain to the tyrosine phosphorylation site of the receptor complex, at which point JAK kinase is spatially adjacent...
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Figure 5. Inhibition of miR-203 expression attenuates ovarian cancer cell proliferation and induces apoptosis. A, QRT-PCR detection of gene expression. B, Western blot detection of protein expression. C, Flow detection of cell proliferation. D, Flow detection of apoptosis. *Represents $p < 0.05$ compared to miR-NC.
to STAT and phosphorylates its hydroxytyrosine site, phosphorylated and activated STAT is separated from the receptor complex, forms a dimer and transports from the cytoplasm to the nucleus, acts on specific DNA fragments, and regulates gene transcription and expression.\textsuperscript{2,17,18} SOCS3 is one of the most important and widely expressed members of the SOCS family. SOCS3 directly inhibits JAK kinase activity and STAT phosphorylation, and inhibits the activity and transmission of JAK-STAT signaling pathway\textsuperscript{4,6}. As an important tumor suppressor, the expression or decreased function of SOCS3 facilitates the occurrence, progression, and metastasis of various tumors\textsuperscript{19-21}. Authors\textsuperscript{5-7} have found that, as a tumor suppressor gene, abnormal expression or functioning of SOCS3 is associated with the occurrence, progression, metastasis, and prognosis of ovarian cancer.

Several researches have indicated that the expression and function of miR-203 are abnormally altered in gastric cancer\textsuperscript{22}, intestinal cancer\textsuperscript{23}, breast cancer\textsuperscript{1}, and other tumors. This study investigated the role miR-203 plays in terms of regulating SOCS3 expression and affecting JAK2-STAT3 pathway activity in ovarian cancer cell proliferation and apoptosis.

The results of qRT-PCR showed that compared with adjacent tissues, the expression of miR-203 was significantly increased in tumor tissues of ovarian cancer patients, while the expression of SOCS3 mRNA was significantly decreased, suggesting that the expression of miR-203 could play a role in reducing the expression of SOCS3 and promoting the pathogenesis of ovarian cancer. Survival analysis showed that the survival rate and prognosis of patients with high expression of miR-203 were significantly worse than those with lower expression. miR-203 mimic significantly reduced the relative Luciferase activity in HEP3B cells transfected with pGL3-SOCS3-WT, indicating that there is a targeted regulatory relationship between miR-203 and SOCS3 mRNA. In this study, the results of cell culture in vitro showed that compared with normal ovarian epithelial IOSE80 cells, the expression of miR-203 in ovarian cancer HO8910 and SKOV3 cells was significantly increased, while the expression of SOCS3 mRNA was significantly decreased, which further suggested an increase in the expression level of miR-203 plays a role in promoting the expression of SOCS3 and promoting the onset of ovarian cancer. In this study, miR-203 inhibitor or miR-203 mimic was transfected into ovarian cancer cells cultured in vitro, and the effects of ovarian cancer cells were observed to further investigate the role of miR-203 in ovarian cancer. The results of this study showed that transfection of miR-203 inhibitor significantly up-regulated the expression of SOCS3 mRNA and protein in ovarian cancer cells and weakened the expression of JAK2 and STAT3 protein. The cell proliferation ability was significantly weakened, and the apoptosis was significantly increased. On the other hand, transfection of miR-203 mimic overexpressing miR-203 promoted the proliferation of ovarian cancer cells and weakened the apoptosis of cells, confirming that miR-203 plays a role as a cancer-promoting gene in ovarian cancer. In the study of the relationship between miR-203 and ovarian cancer, the results of the study by Shao et al\textsuperscript{24} showed that abnormal expression of miR-203 is associated with poor prognosis of various tumors such as pancreatic cancer, colorectal cancer, and ovarian cancer. The risk ratio for poor prognosis with ovarian cancer was 1.85 (95% CI 1.45 to 2.37). Azizmohammadi et al\textsuperscript{12} found that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues, and the expression of miR-203 was abnormally increased and was associated with FIGO stage ($p=0.006$), pathological grade ($p=0.03$). The high expression level of miR-203 was associated with poor prognosis ($p=0.001$). Wang et al\textsuperscript{14} showed that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues, and the expression of miR-203 was abnormally increased and associated with FIGO stage ($p<0.001$), pathological grade ($p=0.02$), lymph node metastasis ($p<0.001$), and recurrence ($p<0.001$), and the expression of miR-203 increased with the overall survival rate of patients ($p<0.001$) and no disease progression survival rate was associated with a decrease ($p<0.001$). Xiaohong et al\textsuperscript{11} disclosed that the expression of miR-203 was significantly increased in tumor tissues of patients with ovarian cancer compared with adjacent tissues. Overexpression of miR-203 promoted proliferation and cloning formation, and migration of ovarian cancer cells Ovca429...
and Ovca433. Down-regulation of miR-203 expression can attenuate the proliferation, clonality, and migration of ovarian cancer Ovca429, Ovca433 cells. The biological effect of miR-203 on ovarian cancer cells is by targeting the inhibition of Pyruvate Dehydrogenase E1 Beta Subunit (PDHB) gene expression and is achieved by regulating the glycolytic pathway of ovarian cancer cells. In this study, the expression of miR-203 was associated with ovarian cancer, and miR-203 plays a role in the role of oncogenes in ovarian cancer. This study combines the targeted regulatory relationship between miR-203 and SOCS3, revealing that miR-203 plays a role in regulating SOCS3 expression, affecting JAK-STAT pathway activity, and cell proliferation and apoptosis of ovarian cancer cells. This has not been reported in any domestic and foreign research. However, whether miR-203 regulates the biological effects of SOCS3 on ovarian cancer cells in vivo is unclear and needs to be confirmed by further animal experiments.

Conclusions

The increased expression of miR-203 and the decreased expression of SOCS3 are related to the pathogenesis of ovarian cancer. MiR-203 can inhibit the proliferation of ovarian cancer cells by targeting the inhibition of SOCS3 expression and affecting the activity of JAK/STAT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


