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MiR-203 regulates JAK-STAT pathway in affecting pancreatic cancer cells proliferation and apoptosis by targeting SOCS3

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Abstract. – **OBJECTIVE:** **Janus kinase (JAK)-signal transducer and transcriptional activator (STAT) pathway overactivation is closely related to tumorigenesis. Cytokine signal transduction inhibitor 3 (SOCS3) is a negative regulator of JAK-STAT. It is shown that miR-203 is significantly elevated in the pancreatic cancer tissues. The bioinformatics analysis revealed a targeted binding site between miR-203 and the 3'-UTR of SOCS3 mRNA. This study investigated the role of miR-203 in regulating SOCS3 expression and the proliferation and apoptosis of the pancreatic cancer cells.**

PATIENTS AND METHODS: **Quantitative Real Time-PCR (qRT-PCR) was used to detect the expressions of miR-203 and SCOS3 mRNA in tumor tissues and paracancerous tissues. The Dual-Luciferase reporter gene assay was adopted to validate the target interaction between miR-203 and SOCS3. The PANC-1 cells were cultured *in vitro* and divided into miR-NC group and miR-203 inhibitor group followed by an analysis of the expressions of SOCS3, p-JAK2, and p-STAT3, cell apoptosis by flow cytometry, and cell proliferation by EdU staining.**

RESULTS: **Compared with the adjacent tissues, miR-203 expression was significantly increased, while SOCS3 mRNA level was significantly declined in the tumor tissues of pancreatic cancer patients. There was a targeted regulatory relationship between miR-203 and SOCS3 mRNA. Compared with those in HPDE6-C7 cells, miR-203 level was upregulated, whereas SOCS3 mRNA and the protein expressions were reduced in pancreatic cancer PANC-1 and BXPC3 cells. The transfection of miR-203 inhibitor significantly increased SOCS3 mRNA and the protein levels, decreased p-JAK2 and p-STAT3 protein expressions, enhanced cell apoptosis, and inhibited cell proliferation in the pancreatic cancer PANC-1 cells.**

CONCLUSIONS: **Increased miR-203 expression and reduced SOCS3 level are associated with the pathogenesis of pancreatic cancer. MiR-203 can regulate the proliferation and apoptosis of the pancreatic cancer cells by targeting the inhibited SOCS3 expression and regulating the JAK-STAT pathway activity.**

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

Introduction

Pancreatic carcinoma (PC) is a common malignant tumor of the digestive system. Its therapeutic effect and prognosis are extremely poor with a high mortality rate. The death caused by pancreatic cancer accounts for the top 5 of cancer related mortality rates[1].

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway are widely found in various tissues and cells. The JAK-STAT pathway is involved in a variety of biological processes, such as cell survival, proliferation, migration, and invasion[2,3]. SOCS3 is one of the most common members of the cytokine signaling (SOCS) family and directly inhibits the activity of JAK kinase and phosphorylation of its downstream STAT protein, thus blocking JAK-STAT signaling pathway activation[4]. As an important tumor suppressor, the expression or the impaired function of SOCS3 plays a key role in the occurrence, progression, and metastasis of various tumors[5-7]. It is found that the abnormal expression or dysfunction of SOCS3 plays a crucial role in the regulation, progression, and metastasis of pancreatic cancer[8-10].

MiR-203 is a well-studied microRNA that is associated with the occurrence, progression, drug resistance, and prognosis of various tumors[11-13].

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Currently, it was observed that the increased miR-203 expression is closely related to the occurrence and development of pancreatic cancer, suggesting that miR-203 may be a cancer-promoting factor in the pathogenesis of pancreatic cancer[14,15]. The bioinformatics analysis revealed a targeted binding site between miR-203 and the 3'-UTR of SOCS3 mRNA. This study investigated the role of miR-203 in regulating SOCS3 expression and the proliferation and apoptosis of the pancreatic cancer cells.

Patients and Methods

Main Reagents and Materials

The human normal pancreatic epithelial cells HPDE6-C7 and HEK293T were purchased from Hunan Fenghui organism (Changsha, China). The pancreatic cancer cells PANC-1 and BXPC3 were purchased from Hangzhou Yinuo Biotechnology Co., Ltd (Hangzhou, China). The Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and Opti-MEM medium were purchased from Gibco (Grand Island, NY, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA). The PrimeScript™ RT reagent Kit and SYBR reagent were purchased from TaKaRa (Ostu, Shiga, Japan). MiR-203 inhibitor, miR-NC, and miR-203 mimic were provided by RiboBio (Guangzhou, China). The rabbit anti-human polyclonal p-STAT3 antibody was purchased from CST (Danvers, MA, USA). The rabbit anti-human polyclonal p-JAK2 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The mouse anti-human polyclonal SOCS3 antibody was purchased from Abcam (Cambridge, MA, USA). The mouse anti-human polyclonal β-actin antibody and HRP-conjugated secondary antibody were purchased from Sangon Biotechnology (Shanghai, China). pGL3 vector and the Dual-Luciferase activity assay kit were purchased from Promega (Madison, WI, USA). Annexin V-FITC/PI double-stained cell apoptosis assay reagent was purchased from Nanjing Kaiji (Nanjing, China). The EdU-Alexa Fluor 488 cell proliferation assay kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The BCA protein quantification kit was purchased from Beyotime (Nantong, China).

Clinical Information

42 pancreatic cancer patients who were treated in our hospital from February 2018 to September 2018 were enrolled, including 25 males and 17 females with an average age of 48.9±13.7 years. The tumor tissue and adjacent tissues at least 2 cm away from the tumor during the operation were collected. All the tissue specimens were diagnosed by the pathological examination. The patients signed an informed consent and the study was approved by the Ethics Committee of the hospital.

Cell Culture

HPDE6-C7, PANC-1, and BXPC3 cells were inoculated in DMEM medium containing 10% FBS and 1% streptomycin at 5% CO_2 at 37°C. The cells were passaged at 1:4 or 1:5 and used for experiments at logarithmic growth phase.

Dual-Luciferase Gene Reporter Assay

The PCR product of the SOCS3 3'-UTR full-length fragment or mutant fragment was double-digested and then ligated into the pGL3 vector. After sequencing, the plasmid was designated as pGL3-SOCS3-wt and pGL3-SOCS3-MUT. The HEK293T cells were transfected with pGL3-SOCS3-WT (or pGL3-SOCS3-MUT) together with miR-203 mimic (or miR-NC) by Lipofectamine 2000. After incubation for 48 h, the relative Luciferase activity was detected by the Dual-Luciferase reporter assay system kit according to the manual.

Cell Transfection

PANC-1 cells were cultured *in vitro* and divided into miR-NC transfection group and miR-203 inhibitor transfection group. 10 µL Lipofectamine 2000, 50 nmoL miR-NC, and 50 nmoL miR-203 inhibitor were diluted with 100 µL of serum-free Opti-MEM at room temperature for 5 min, respectively. Next, Lipofectamine 2000 was mixed with miR-NC and miR-203 inhibitor at room temperature for 20 min, respectively. At last, they were added to the cell culture medium and incubated for 72 hours.

qRT-PCR

The total RNA was extracted using TRIzol reagent and reversely transcripted into cDNA using the PrimeScript™ RT reagent Kit. The qRT-PCR PCR was performed on Bio-Rad CFX96 (Hercules, CA, USA) in a system consisting of 2×SYBR Green Mixture 5.0 µL, Forward Primer (2.5 µM) 0.5 µL, Reverse Primer (2.5 µM) 0.5 µL, cDNA 1 µL, and RNase Free dH_2O with reaction conditions as follows: 95°C for 5 min, followed by

40 cycles of 95°C for 15 s and 60°C for 1 min. The primer sequences were: miR-221 (Forward): 5'-ACACTCCAGCTGGGAGCTACATTGTCT-GCTGG-3'; miR-221 (Reverse): 5'-CTCAACT-GGTGTCGTGGA-3'; U6 (Forward): 5'-CTC-GCTTCGGCAGCACA-3'; U6 (Reverse): 5'-AACGCTTCACGAATTTGCGT-3'. SOCS3 (Forward): 5'-GTCCCCCCAGAAGAGCCTAT-TA-3'; SOCS3 (Reverse): 5'-TTGACGGTCTTC-CGACAGAGAT-3'. GAPDH (Forward): 5'-TG-CACCACCAACTGCTTAGC-3'; GAPDH (Reverse): 5'-GGCATGGACTGTGGTCATGAG-3'. The relative expression of the target mRNA was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western Blot

The total protein was extracted from the cells using the RIPA lysis buffer. After being quantified by BCA method, a total of 40-μg proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane at 300 mA for 90 min. After blockage with 5% skim milk at room temperature, the membrane was incubated with primary antibody at 4°C overnight (SOCS3, p-JAK2, p-STAT3, and β-actin at 1:2000, 1:800, 1:800, and 1:15000, respectively). After being washed by Phosphate-Buffered Saline and Tween 20 (PBST), the membrane was further incubated with HRP conjugated secondary antibody at room temperature for 60 min (1:10000) followed by development and exposure after addition of enhanced chemiluminescence (ECL) reagent.

Cell Apoptosis Detection

The cells were digested by trypsin and then collected. After resuspended in 100 μl binding buffer, the cells were added with 5 μl Annexin V-FITC and 5 μl PI at room temperature away from light for 15 min. Then, the cell apoptosis was measured by flow cytometry.

EdU Staining

The cells were added with EdU solution at 10 μM in logarithmic phase. After incubation for 2 h, the cells were seeded for 48 h and digested by trypsin. After being fixed in paraformaldehyde, the cells were incubated with 100 μL TritonX-100 at room temperature and in 500 μL reaction fluid at room temperature away from light for 30 min. At last, cell proliferation was analyzed on FC 500 MCL flow cytometry.

Statistical Analysis

The Statistical Product and Service Solution (SPSS 18.0; SPSS Inc., Chicago, IL, USA) was applied for data analysis. The measurement data was presented as mean ± standard deviation (SD) and was compared by Student *t*-test. $p < 0.05$ was depicted as significant difference.

Results

MiR-203 Expression Increased, While SOCS3 Level Declined in PC Tissue

qRT-PCR showed that the expression of miR-203 was significantly increased in the tumor tissues of PC patients compared with the adjacent tissues (Figure 1A). Moreover, qRT-PCR exhibited that the expression of SOCS3 mRNA in tumor tissues of PC patients was significantly lower than that in the adjacent tissues (Figure 1B).

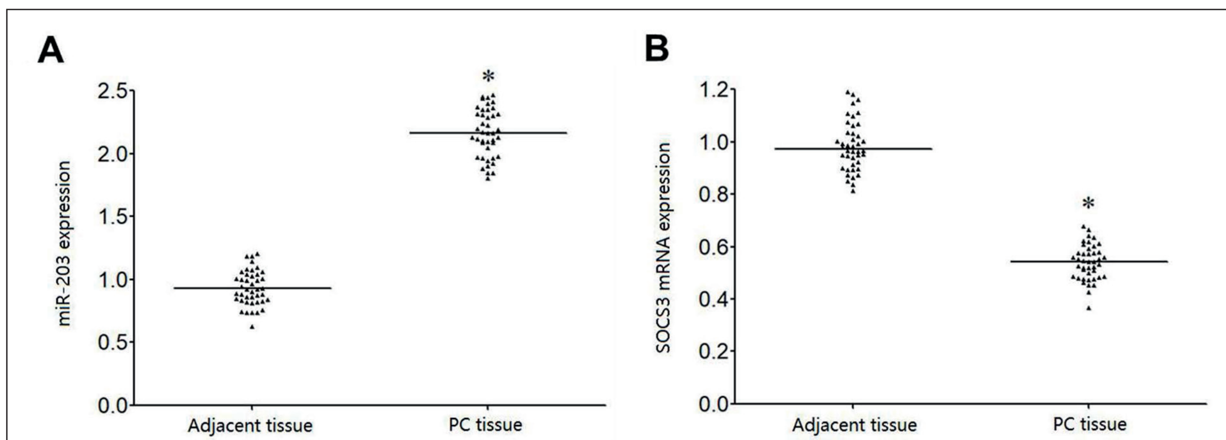

Figure 1. MiR-203 expression increased, while SOCS3 level declined in PC tissue. ***A,*** qRT-PCR detection of miR-221 expression. ***B,*** qRT-PCR detection of SOCS3 mRNA expression. *$p < 0.05$, compared with adjacent tissue.

The Targeted Regulatory Relationship Between miR-203 and SOCS3 mRNA

The bioinformatics analysis showed the complementary binding site between miR-203 and the 3'-UTR of SOCS3 mRNA (Figure 2A). The Dual-Luciferase reporter gene assay exhibited that miR-203 mimic transfection significantly reduced the relative Luciferase activity in HEK293T cells transfected by pGL3-SOCS3-WT but not by pGL3-SOCS3-MUT, confirming that SOCS3 was the target gene of miR-203 (Figure 2B).

MiR-203 and SOCS3 Abnormal Expressions in PC Cells

qRT-PCR demonstrated that, compared with HPDE6-C7 cells, the expression of miR-203 was significantly upregulated, while the expression of SOCS3 mRNA was significantly decreased in PANC-1 and BXPC3 cells (Figure 3A-B). The Western blot analysis revealed that the expression of SOCS3 protein in PANC-1 and BXPC3 cells was significantly lower than that of HPDE6-C7 cells (Figure 3C).

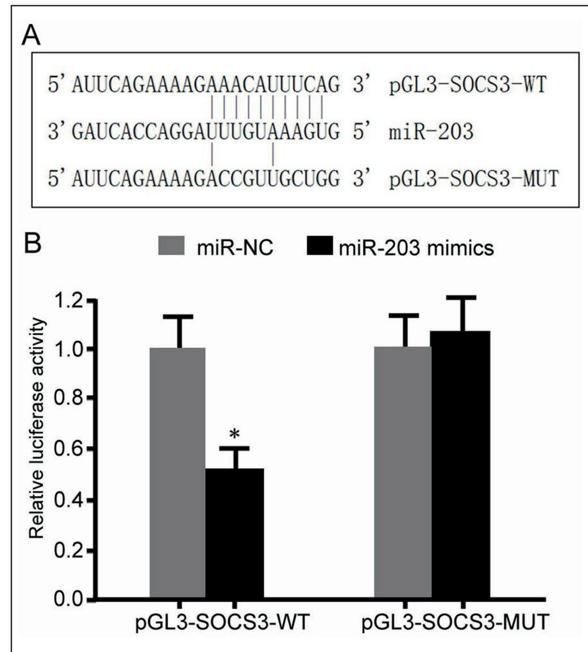

Figure 2. The targeted regulatory relationship between miR-203 and SOCS3. ***A,*** The complementary binding site between miR-203 and the 3'-UTR of SOCS3 mRNA. ***B,*** Dual-Luciferase reporter assay. *$p < 0.05$, compared with miR-NC.

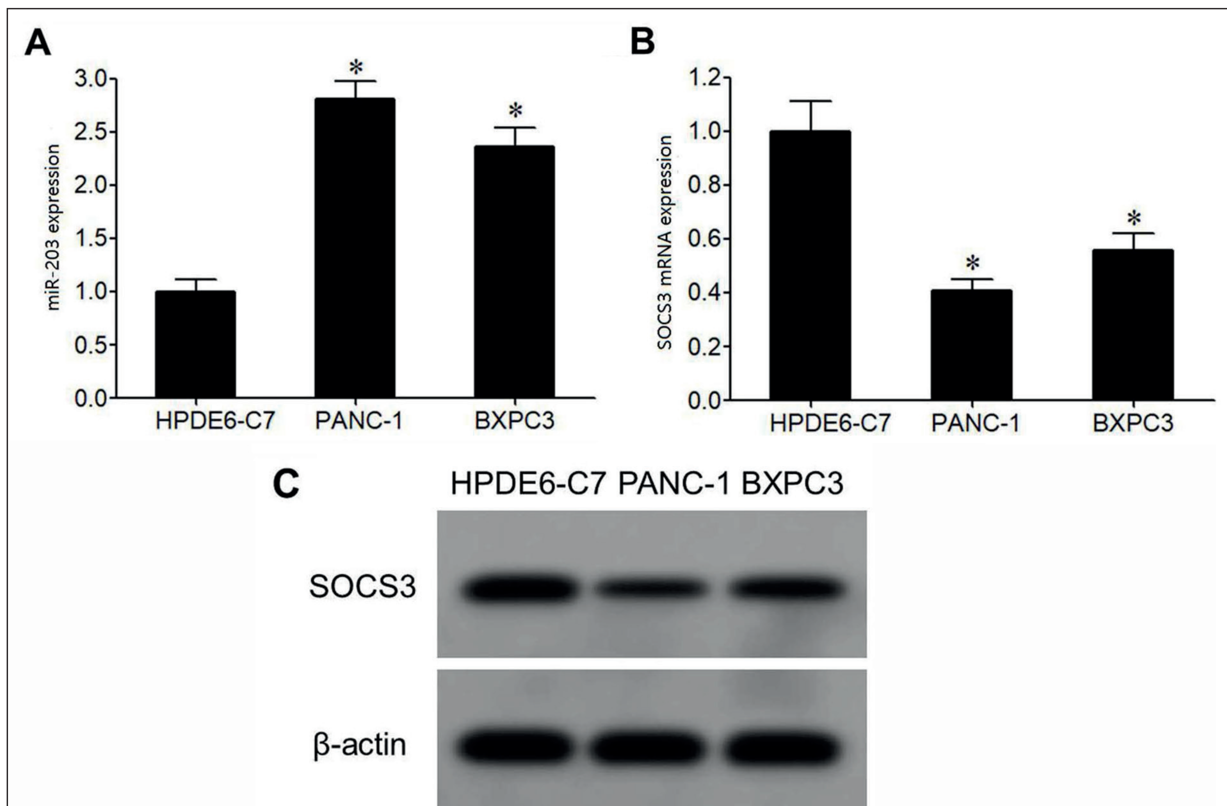

Figure 3. MiR-203 and SOCS3 abnormal expressions in PC cells. ***A,*** qRT-PCR detection of miR-203. ***B,*** SOCS3 mRNA expression. ***C,*** Western blot detection of SOCS3 protein expression. *$p < 0.05$, compared with HPDE6-C7.

MiR-203 Suppression Promoted PC Cell Apoptosis and Attenuated Cell Proliferation

qRT-PCR found that the expression of SOCS3 mRNA in PANC-1 and BXPC3 cells was significantly elevated in miR-203 inhibitor transfection group compared with miR-NC transfection group (Figure 4A). The Western blot analysis showed that the transfection of miR-203 inhibitor significantly enhanced the expression of SOCS3 protein, and significantly reduced the p-JAK2 and p-STAT3 protein levels in PANC-1 and BXPC3 cells (Figure 4B). Flow cytometry demonstrated that the number of cell apoptosis was significantly higher, while the cell proliferation ability was significantly weakened in the miR-203 inhibitor transfection group compared with the miR-NC transfection group (Figures 4C and 4D).

Discussion

When a cytokine or growth factor binds to an intracellular receptor as a ligand, the receptor can form a homologous or heterodimer, and phosphorylate the JAK kinase. The activated JAK can phosphorylate the tyrosine residue on the receptor, causing STAT to translocate to the tyrosine phosphorylation site of the receptor complex *via* the SH2 domain. At that point, JAK kinase is spatially adjacent to STAT and phosphorylates its hydroxytyrosine site. Phosphorylated STAT is separated from the receptor complex, forms a dimer, and translocates from the cytoplasm to the nucleus, acts on specific DNA fragments, and regulates the gene transcription and expression[2,16-18]. SOCS3 is one of the most common members of the cytokine signaling inhibitory (SOCS) family

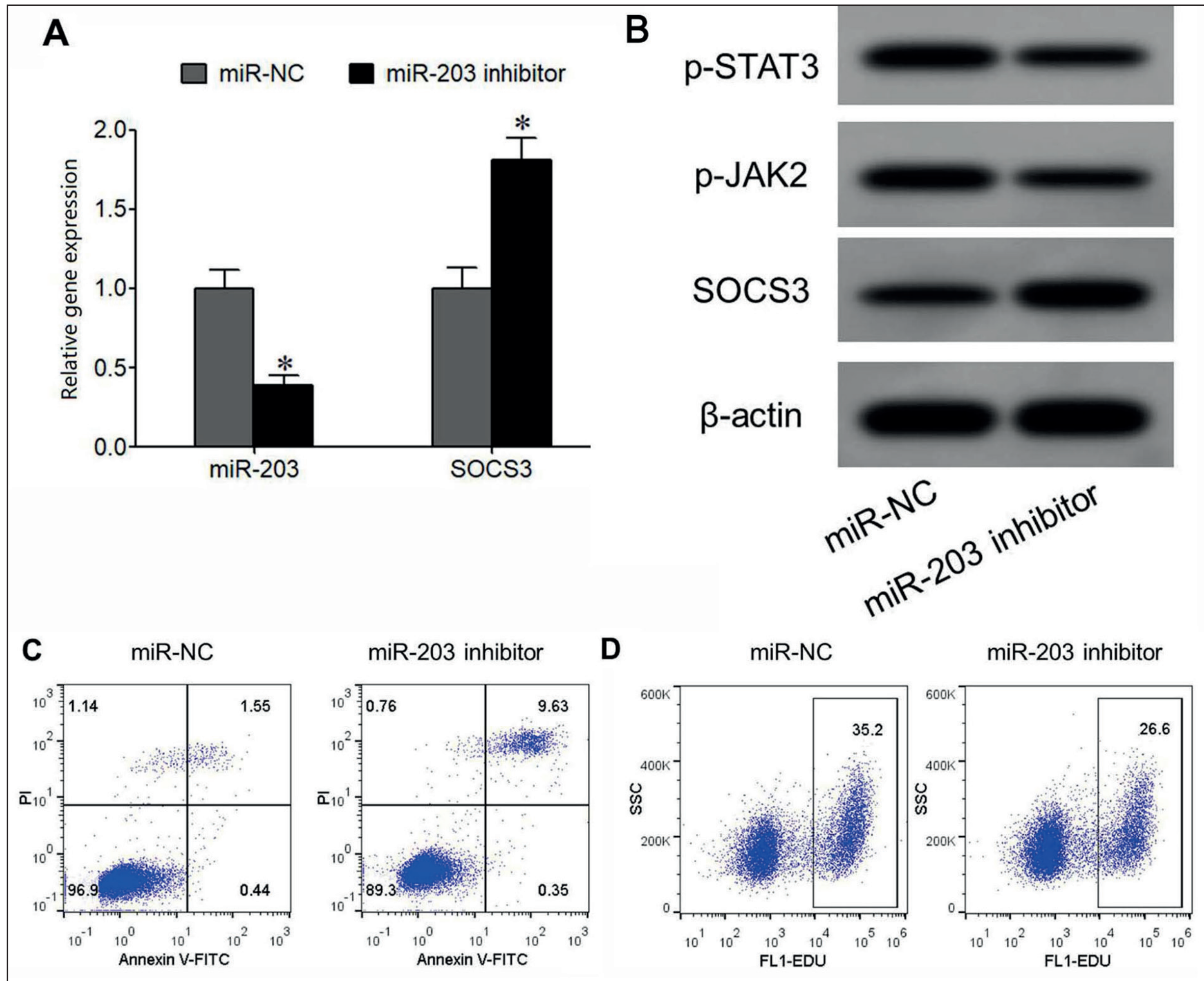

Figure 4. MiR-203 suppression promoted PC cell apoptosis and attenuated cell proliferation. ***A,*** qRT-PCR detection of gene expression. ***B,*** Western blot detection of protein expression. ***C,*** Flow cytometry detection of cell apoptosis. ***D,*** EdU staining detection of cell proliferation. $*p < 0.05$, compared with miR-NC.

and can directly inhibit JAK kinase activity and STAT phosphorylation, and inhibit JAK-STAT signaling pathway[4]. As an important tumor suppressor, the expression or function of SOCS3 plays a catalytic role in the occurrence, progression, and metastasis of various tumors, such as colorectal cancer[19], gastric cancer[6], and thyroid cancer[7]. It is reported that the abnormal expression or dysfunction of SOCS3 plays a crucial role in the occurrence, progression, and metastasis of pancreatic cancer[8-10].

MicroRNA is an endogenous non-coding small-molecule single-stranded RNA of eukaryotes with a length of about 22-25 nucleotides, which complementarily binds to the 3'-UTR of the target gene mRNA, leading to degradation or inhibition of its translation. MicroRNAs, which account for 1% of human genes, regulate the expression of more than 1/3 of human genes[19-21]. The abnormality of miR-203 is related to the occurrence, progression, drug resistance, and prognosis of various tumors, such as prostate cancer[11], bladder cancer[12], and gastric cancer[13]. At present, it is revealed that the abnormal increase of miR-203 expression is closely related to the occurrence and development of pancreatic cancer, suggesting that miR-203 may be a cancer-promoting factor[14,15]. The bioinformatics analysis revealed a targeted binding site between miR-203 and the 3'-UTR of SOCS3 mRNA. This study investigated the role of miR-203 in regulating the SOCS3 expression and affecting the proliferation and apoptosis of the pancreatic cancer cells.

In this study, qRT-PCR analysis showed that, compared with the adjacent tissues, the expression of miR-203 was significantly increased, while the expression of SOCS3 mRNA was significantly declined in the tumor tissues of patients with pancreatic cancer. It suggested that there may be a targeted regulatory relationship, and that the elevated expression of miR-203 may play a role in reducing the SOCS3 level and promoting the pathogenesis of pancreatic cancer. The Dual-Luciferase reporter gene assay exhibited that miR-203 mimic transfection significantly reduced the relative Luciferase activity in HEK293T cells transfected by pGL3-SOCS3-WT but not by pGL3-SOCS3-MUT, confirming that SOCS3 was the target gene of miR-203. In addition, it was found that the expression of miR-203 was significantly higher, while the expression levels of SOCS3 mRNA and protein were significantly lower in pancreatic cancer PANC-1 and BXPC3 cells than that in the normal pancreatic HPDE6-C7 cells, further indicating that the elevated miR-203 and decreased SOCS3 play a regulatory role in the pathogenesis of pancreatic cancer. Zhang et al[22] reported that the expression of miR-203 was significantly increased in tumor tissues of patients with pancreatic cancer, and it was related to poor prognosis. Shi et al[23] demonstrated that the survival and prognosis of patients with higher expression of miR-203 were significantly worse than those with lower expression of miR-203 ($p = 0.0026$). Ali et al[24] showed that, compared with normal pancreatic tissue, the expression of miR-203 was significantly elevated in tumor tissues of patients with pancreatic cancer ($p = 0.0058$), but the expression level of miR-203 was not found to be related to the survival and prognosis of patients ($p = 0.942$). Shao et al[25] revealed that the survival and prognosis of patients with higher expression of miR-203 were significantly worse than those with lower expression of miR-203 (HR=2.18, 95% CI 1.31-2.49, $p = 0.001$). Frampton et al[26] observed that the increased expression of miR-203 is associated with poor survival and prognosis in patients with pancreatic cancer. Greither et al[15] exhibited that the expression of miR-203 was significantly upregulated in tumor tissues of patients with pancreatic cancer compared with normal pancreatic tissue, and it was associated with adverse outcomes (RR=0.942, $p = 0.017$). The above results suggested that the elevated miR-203 is a cancer-promoting factor in the pathogenesis of pancreatic cancer. However, the specific mechanism of miR-203 in the regulation of pancreatic cancer has not been elucidated.

To further investigate the role and mechanism of miR-203 in the regulation of pancreatic cancer cells, this study transfected miR-203 inhibitor to pancreatic cancer cells. The results of this study showed that the transfection of miR-203 inhibitor significantly down-regulated the expression of miR-203, up-regulated the expression of SOCS3 gene and protein, reduced the expression of p-JAK2 and p-STAT3 protein in pancreatic cancer PANC-1 cells, attenuated cell proliferation, and enhanced cell apoptosis. Ren et al[14] showed that miR-203 can act as a tumor-promoting gene in pancreatic cancer to facilitate cell proliferation, migration, and invasion by inhibiting its target gene SIK1, while the overexpression of SIK1 can antagonize the promotion of miR-203 on proliferation, migration, and invasion of the pancreatic cancer cells. Our results observed that

miR-203 can inhibit the expression of SOCS3, affect the activity of JAK-STAT pathway, enhance the malignant biological characteristics of pancreatic cancer cells, and play a role in promoting cancer in pancreatic cancer, which was similar to the results of Ren et al[14]. Currently, there are few studies on the biological effects of miR-203 on pancreatic cancer cells. This study found the targeting regulation between miR-203 and SOCS3, revealing that miR-203 regulates SOCS3 expression and affects JAK-STAT pathway activity to play a role in the pancreatic cancer cell proliferation and apoptosis. However, whether miR-203 regulates SOCS3 and affects the biological characteristics of pancreatic cancer cells *in vivo* is still unclear and requires further investigations in the future.

Conclusions

We found that the increased miR-203 expression and reduced SOCS3 levels are associated with the pathogenesis of pancreatic cancer. MiR-203 can regulate the proliferation and apoptosis of pancreatic cancer cells by targeting the inhibition of SOCS3 expression and regulating JAK-STAT pathway activity.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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