

MiR-221 inhibits proliferation of pancreatic cancer cells via down regulation of SOCS3

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Abstract. – OBJECTIVE: The over-activation of Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway induced by cytokines are closely correlated with tumorigenesis. Suppressor of cytokine signaling 3 (SOCS3) serves as a negative regulator for JAK-STAT, and its down-regulation is involved in the oncogenesis of pancreatic cancer. We aimed at investigating the effect of miR-221 on the expression and proliferation, cycle and apoptosis of pancreatic cancer cells and determine the related mechanism.

PATIENTS AND METHODS: Dual luciferase reporter gene assay was used to analyze the regulation between miR-221 and SOCS3. The expressions of miR-221, SOCS3, p-JAK and p-STAT3 in normal human pancreatic epithelial cell HPDE6-C7 and pancreatic cancer cell PANC-1 were quantified by qPCR and Western blot. Flow cytometry was used to identify cell cycle and proliferation. *In vitro* cultured PANC-1 cells were transfected with miR-221 inhibitor or pIRES2-SOCS3. The expressions of miR-221, SOCS3, p-JAK and p-STAT3, along with the cell proliferation or apoptosis, were compared.

RESULTS: Bioinformatics analysis showed the existence of binding site between miR-221 and 3'-UTR of SOCS3 mRNA. Dual luciferase gene reporter assay confirmed the targeted regulation between miR-221 and SOCS3. Compared to HPDE6-C7 cells, higher levels of miR-221, p-JAK and p-STAT3 expression, and lower expression of SOCS3, were found in PANC-1 cells, along with the increase of cell proliferation. Transfection of miR-221 inhibitor or pIRES2-SOCS3 remarkably enhanced SOCS3 expression, inhibited the levels of p-JAK and p-STAT3 expression, and impeded the proliferation of PANC-1 cells.

CONCLUSIONS: MiR-221 decreases proliferation potency of PANC-1 cells and affects JAK-STAT3 signaling pathway via inhibiting SOCS3.

Key Words

miR-221, SOCS3, JAK-STAT3, Pancreatic cancer, Proliferation, Cell cycle, Apoptosis.

ciency and prognosis. It has high mortality and has become top 5 of deadly cancers¹. Therefore, the investigation of signaling pathway implicated in PC pathogenesis is of critical importance for the improvement of chemotherapy efficiency and guidance for individualized treatment, which benefits patient prognosis.

Janus kinase (JAK)- signal transducer and activator of transcription (STAT) signaling pathway are widely expressed in multiple tissues and cells of mammals, and participate in the regulation of diverse biological processes such as cell survival, proliferation, cycle progression, apoptosis, migration and invasion^{2,3}. Suppressors of cytokine signaling (SOCS) can block phosphorylation of STAT via suppressing JAK kinase activity, thus contributing to the inhibitory function towards transducing potency and activity of JAK-STAT^{4,5}. SOCS3 is profiled as an important tumor suppressor gene, and its down-regulation is correlated with occurrence and progression of multiple tumors. Various studies^{6,7} showed abnormal decrease of SOCS3 expression in PC tissues, and its expression level was correlated with PC progression, tumor growth and distal metastasis. MicroRNA (miR) belongs to the group of endogenous non-coding small molecule single-stranded RNA with 22-25 nucleotide at length in eukaryotes, and can modulate the expression of target gene via complementary binding onto 3'-UTR of its mRNA sequence. Although it takes up 1% of human genes, miR can modulate the expression of more than one third of human genes⁸. Increasing studies showed that miR-221 worked as an oncogene, and its abnormal up-regulation played roles in facilitating occurrence and progression of multiple tumors including breast cancer⁹ and gastric carcinoma¹⁰. Evidence also indicated abnormal rise of miR-221 expression in prostate cancer, and miR-221 was correlated with the development and distal metastasis of PC patients¹¹. This work aims to determine the role of miR-221 on the biological process of PC cells and seeks to find related mechanisms.

Introduction

Pancreatic carcinoma (PC), a commonly occurred malignant tumor, is characterized by an extreme high malignancy, worse treatment effi-

Materials and Methods

Major reagents and materials

Normal human pancreatic epithelial cell line HPDE6-C7 was purchased from ScienCell (Carlsbad, CA, USA). PC cell line PANC-1 was from Gino Biotech (Hangzhou, Zhejiang, China). Roswell Park Memorial Institute-1640 (RPMI)-1640 medium and streptomycin-penicillin were purchased from HyClone (South Logan, UT, USA). Opti-MEM was collected from Gibco (Rockville, MD, USA). Fetal bovine serum (FBS) was acquired from Gemini Bio Products (Shanghai, China). RNA extract reagent Total RNA Kit was obtained from Omega Bio-Tek (Norcross, GA, USA). DNA extraction kit EasyPure Blood Genomic DNA Kit was bought from Quanshijin Biotech (Beijing, China). Fluorescent quantitative PCR reagent PrimerScript RT reagent Kit was purchased from TaKaRa (Kusatsu, Otsu, Shiga, Japan). Annexin V/PI apoptosis kit was from Biolegend (San Diego, CA, USA). Cell proliferation test reagent Click-iT EdU Flow Cytometry Assay Kit was obtained from Molecular Probes (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase activity assay kit Dual-Glo Luciferase Assay System was collected from Promega (Madison, WI, USA). Luciferase reporter plasmid pLUC was from BioVector (Beijing, China). Transfection reagent Lip 2000 was bought from Invitrogen (Carlsbad, CA, USA). Mouse anti-human JAK, p-JAK, STAT3 and p-STAT3 antibody were purchased from CST (Danvers, MA, USA). Rabbit anti-human SOCS3 and β -actin antibody were obtained from Abcam (Cambridge, MA, USA). Horseradish-peroxidase (HRP) conjugated secondary antibody was obtained from Sango Bio (Shanghai, China). MiR-221 mimic, miR-221 inhibitor and miR-NC were purchased from RioBio (Guangzhou, Guangdong, China).

Cell culture

HPDE6-C7 and PANC-1 cells were all incubated in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin, and were incubated in 37°C chamber with 5% CO₂. Culture medium was changed every three days. Cells were passed when reaching 70-80% confluence at 1:4 ratio. Those cells at log-growth phase with satisfactory status were used for experiments.

Luciferase reporter gene assay

PANC-1 cells were digested by trypsin and were collected. Genomic DNA was extracted

using EasyPure Blood Genomic DNA Kit following the manual instruction. Using DNA as the template, full-length fragment or fragment containing mutant sequent of 3'-UTR of SOCS3 gene was amplified. PCR products were extracted from agarose gel, and were ligated into pLUC plasmid, which was used to transform DH5 α competent cells. Bacterial colony with positive screening was amplified and sequenced. Those with correct sequence were selected and were named as pLUC-SOCS3-wt, or pLUC-SOCS3-mut. Lipofectamine 2000 was used to co-transfect pLUC-SOCS3-wt or pLUC-SOCS3-mut with miR-221 mimic or miR-NC into HEK293T cells. After 48 h incubation, Dual-Glo Luciferase Assay System was used to measure the relative luciferase activity.

Construction of SOCS3 over-expression plasmid

CDS domain fragment of SOCS3 gene was amplified, and the target fragment length was determined by agarose gel electrophoresis, with 1640 bp length. After enzymatic digestion with XhoI and BamH I, the fragment was ligated into pIRES2 plasmid, which was used to transform competent cell JM109. Positive clones were selected by ampicillin resistance. Plasmids were then extracted and sequenced to determine the correct insertion of SOCS3 gene fragment. Those with successful gene insertion were named as pIRES2-SOCS3. Empty plasmid pIRES2-Blank was used as the control group.

Grouping of transfected cells

Cultured PANC-1 cells were divided into 4 groups: miR-NC group, miR-221 inhibitor group, pIRES2-Blank group, and pIRES2-SOCS3 group. All these nucleotide fragments and Lipo 2000 were mixed well in Opti-MEM, and incubated in room temperature for 5 min. Nucleotide fragments were then mixed with Lipo 2000 for gentle mixture, and were incubated for 30 min at room temperature. Original medium of PANC-1 cells was removed, and cells were rinsed twice in phosphate-buffered saline (PBS) to remove the serum. Opti-MEM with serum-free was used, and transfection mixture was added into the culture medium. Cells were incubated for 6 h, and RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin was used. After continuous incubation for 72 h, cells were digested by trypsin for collection and for assays of related indexes.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for gene expression

PrimerScript RT reagent Kit was used in qRT-PCR assay to detect relative expression level of genes using RNA extracted by Total RNA Kit.

Reverse transcription system consisted

oligdT Primer (50 μM)	0.5 μL
Random 6 mers (100 μM)	0.5 μL
PrimeScript RT Enzyme Mix	0.5 μL
RNA	1.0 μg
5×PrimeScript Buffer	2 μL
RNase Free dH ₂ O	Up to 10.0 μL

qPCR system consisted

SYBR Fast qPCR Mix	10.0 μL
Forward Primer (10 μM)	0.8 μL
Reverse Primer (10 μM)	0.8 μL
cDNA	2.0 μL
RNase Free dH ₂ O	6.4 μL

Reaction parameters were: 95°C pre-denaturing for 10 min, followed by 40 cycles each containing 95°C denaturing for 10 s, 60°C annealing for 20 s and 72°C elongation for 15 s. PCR was performed on Bio-Rad CFX96 Real-time PCR detection system. Primer sequences were: miR-221P_F: 5'-GTTGG TGGGA GCTAC ATTGT CTGC-3'; miR-221P_R: 5'-GTGTC GTGGA CTCGG CAATT C-3'; U6P_F: 5'-ATTGG AACGA TACAG AGA-AG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; SOCS3P_F: 5'-CCTGC GCCTC AAGAC CTTC-3'; SOCS3P_R: 5'-GTCAC TGC GC TCCAG TAGAA-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'.

Western Blot

Sodium dodecyl sulfate (SDS) lysis buffer was added to lyse cells, and then was boiled for 5 min. Protein concentration was measured and 40 μg samples were loaded onto 8-10% separating gel and 5% condensing gel. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, which was blocked in 5% defatted milk powder for 60 min at room temperature. Primary antibody were anti-SOCS3 at 1:1000, anti-p-JAK at 1:1000, anti-JAK at 1:2000, anti-p-STAT3 at 1:1000, anti-STAT at 1:2000, and anti-β-actin at 1:10000. The membrane was then used in phosphate-buffered saline and Tween (PBST) for three times, followed by room temperature incubation using horseradish-peroxidase (HRP) conjugated secondary antibody (1:10000) for 60 min. The membrane was

washed in PBST for three times, and enhanced chemiluminescence (ECL) approach was used to test protein expression level.

Cell proliferation assay

Transfected cells from all groups were re-suspended and cultured in 10 μM EdU for 120 min. Cells were inoculated into 6-well plate for 48 h continuous culture. Trypsin was then used to digest and collect all cells, which were fixed for 20 min at room temperature. Cells were washed in wash buffer by centrifuge, and were permeable for 15 min at room temperature. 500 μL reaction buffer were added for 30 min in dark incubation at room temperature, followed by one time of wash buffer centrifugation. Cells were eventually re-suspended in 500 μL wash buffer, and were measured for proliferation using Beckman FC500 MCL flow cytometry (Brea, CA, USA).

Flow cytometry for cell cycle

Transfected cells from all groups were digested and collected. After washing in PBS, cells were fixed in 70% ethanol at -20°C for 60 min. Cells were washed twice by centrifugation, and were mixed with propidium iodide (PI) staining buffer containing RNase A for 30min dark incubation at 4°C. Cell cycle was measured by Beckman FC500 MCL flow cytometry apparatus.

Flow cytometry for cell apoptosis

Cells were collected by centrifugation. After washing twice in phosphate-buffered saline (PBS), 100 μL Binding Buffer were added for mixture. 5 μL Annexin V-FITC and 5 μL PI were subsequently added for 10 min dark incubation. 400 μL Binding Buffer were then added to re-suspend cells, which were detected using Beckman FC 500 MCL flow cytometry.

Spectrometry for Caspase-3 activity

Following the manual instruction of Caspase-3 activity assay kit, pNA standard samples were firstly prepared to measure A405 and to plot standard curves. Cells were digested by trypsin and were collected, lysed on ice and centrifuged. The supernatant was saved and transferred to new pre-cold tubes. Assay buffer, test samples, and Ac-DEVD-pNA, were added sequentially into 96-well plate, for 2 h incubation at 37°C. Absorbance value A405 was measured using a microplate reader when founding significant color change, to reflect Caspase-3 activity of test samples.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented by mean±standard deviation (SD). Student *t*-test was used for comparing measurement data between two groups. The comparison of measurement data among multiple groups was performed using one-way analysis of variance (ANOVA) followed by Least Significance Difference (LSD). A statistical significance was defined when $p < 0.05$.

Results

Targeted regulation between miR-221 and SOCS3

Online prediction by microRNA.org showed complementary binding sites between miR-221 and 3'-UTR of SOCS3 mRNA sequence (Figure 1A). Dual luciferase gene reporter assay found that, compared to miR-NC group, miR-221 mimic transfection HEK283T cells gave rise to remarkable decrease in relative luciferase activity. There was no significant change on relative luciferase activity after co-transfection of miR-221 mimic and pLUC-SOCS3-mut into HEK293T cells (Figure 1B), suggesting a targeted regulatory relationship between miR-221 and SOCS3.

Abnormal expression of miR-221 and SOCS3 in PC cells

The result of flow cytometry revealed that the ratio of S phase and G2/M phase of PANC-1 cells significantly increased compared to that in HPDE6-C7 cells, whilst G0/G1 ratio was significantly lower (Figure 2A). Comparing to that in HPDE6-7 cells, positive rate of EdU in PANC-1 cells was significantly elevated, indicating strong proliferation potency (Figure 2B). Moreover, qRT-PCR results showed that, compared to that

in HPDE6-C7 cells, significantly high miR-221 expression with decrease of SOCS3 mRNA level was found in PANC-1 cells (Figure 2C). Western blot results also showed that, compared to that in HPDE6-C7 cells, the SOCS3 protein expression was remarkably reduced in PANC-1 cells, along with the growing expression of p-JAK and p-STAT3 (Figure 2D).

miR-221 down-regulation effectively inhibited PC cell PANC-1 proliferation and induced cell apoptosis

We determined the effect of miR-221 on the development of PC by using miR-221 inhibitor. Our data demonstrated that, compared to miR-NC transfection group, in PANC-1 cells treated with miR-221 inhibitor, the level of SOCS3 was significantly elevated, whilst p-JAK and p-STAT3 expressions were remarkably decreased (Figure 3A-B). Flow cytometry showed that the inhibition of miR-221 significantly decreased proliferation potency (Figure 3C), and remarkably enhanced cell apoptosis level (Figure 3D). We also evaluated the role of SOCS3 by over expression of SOCS3 *in vitro*; the results illustrated that the elevation of SOCS3 expression significantly down regulated the levels of p-JAK and p-STAT3 (Figure 3A and 3B), which enhanced proliferation potency (Figure 3C) and suppressed cell apoptosis (Figure 3D).

Discussion

It is estimated that PC incidence has reached 8.3 per 100 000 people worldwide, with mortality rate up to 7.8%¹². In China, about 91 000 patients were newly diagnosed as PC annually, accompanied with 79 000 cases of death¹³. Generally, PC represents a type of cancers with worse progn-

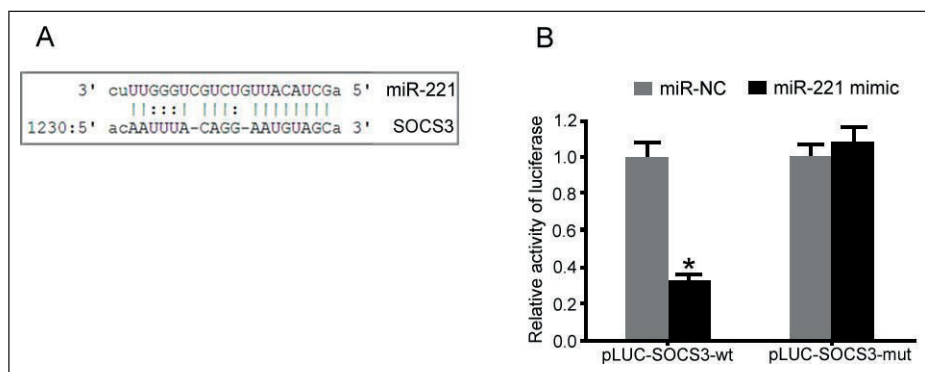
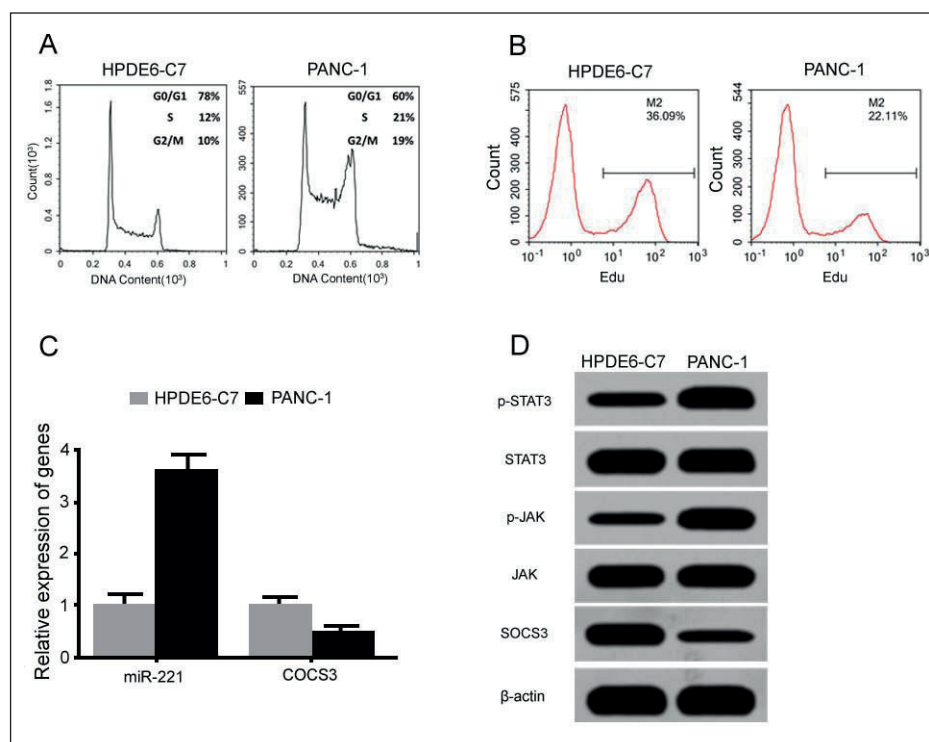


Figure 1. Targeted regulatory relationship between miR-221 and SOCS3. **A**, Schematic diagram for binding sites between miR-221 and SOCS3 mRNA. **B**, Dual luciferase gene reporter assay. *, $p < 0.05$ compared to miR-NC.

Figure 2. Abnormal expression change of miR-221 and SOCS3 in PC cells. **A**, Flow cytometry for cell cycle; **B**, Flow cytometry for cell proliferation potency; **C**, qRT-PCR for gene expression; **D**, Western blot for protein expression. *, $p < 0.05$ compared to HPDE6-C7 cells.



sis, an average survival age around 5 months, and a 5-year survival of about 1-5%^{14,15}. It has been demonstrated that abnormal activation of JAK-STAT signaling pathway plays important regulatory roles in facilitating abnormal proliferation of cells, accelerating cell cycle progression, enhancing invasion or metastasis, and decreasing apoptosis^{2,3}. Accumulative evidence presented that activated STAT detached from receptor complex, formed dimer for the further transportation from cytoplasm into nucleus, where it functions on specific DNA sequence to mediate gene transcription and expression^{2,16,17}. The specific process is further closely correlated with tumor pathogenesis, progression and prognosis. Several studies illuminated abnormal change of JAK-STAT signaling pathway activity in various tumor tissues including prostate cancer¹⁸, colorectal carcinoma⁴, and lung cancer¹⁹. This investigation showed that, compared to normal pancreatic cell HPDE6-C7, PC cell line PANC-1 showed p-JAK and p-STAT3 expressions were remarkably enhanced, with the increase of cell proliferation activity, which suggested the involvement of JAK-STAT signaling pathway in the occurrence and development of PC.

SOCS3 can suppress JAK binding with receptor via its KIR domain, thus suppressing JAK phosphorylation or kinase activity. Alternatively,

it can inhibit JAK phosphorylation on STAT via competitive binding with STAT, thus exerting negative regulatory role on JAK-STAT3 signal pathway activity^{20,21}. Recent investigations shed light on the correlation of its down-regulation with the occurrence and progression of multiple tumors including breast cancer²⁰, colorectal carcinoma²² and prostate cancer²¹. Particularly, abnormal decrease of SOCS3 expression was validated in PC, and its expression level was related with PC progression, tumor growth and distal metastasis^{6,7}. Consistently, our study showed that the over expression of SOCS3 by transfection with pIRES2-SOCS3 decreased p-JAK or p-STAT3 expressions in PANC-1 cells, accompanied with weakened proliferation and potentiated apoptosis of cells, indicating the inhibitory role of SOCS3 in JAK-STAT3 signal pathway as well as PC.

MicroRNAs take a critical part in various malignant tumors²³. MiR-221 is widely reported to have pro-tumor role, and plays facilitating role in occurrence and progression of various tumors. For example, Li et al⁹ showed that miR-221 up-regulation could facilitate proliferation, migration and invasion of breast cancer cells via targeted regulation on PTEN/AKT signaling pathway. Effatpanah et al¹⁰ found significantly higher miR-221 expression in gastric cancer tissues compared to normal gastric mucosa. Li et al²⁴ reported that compared

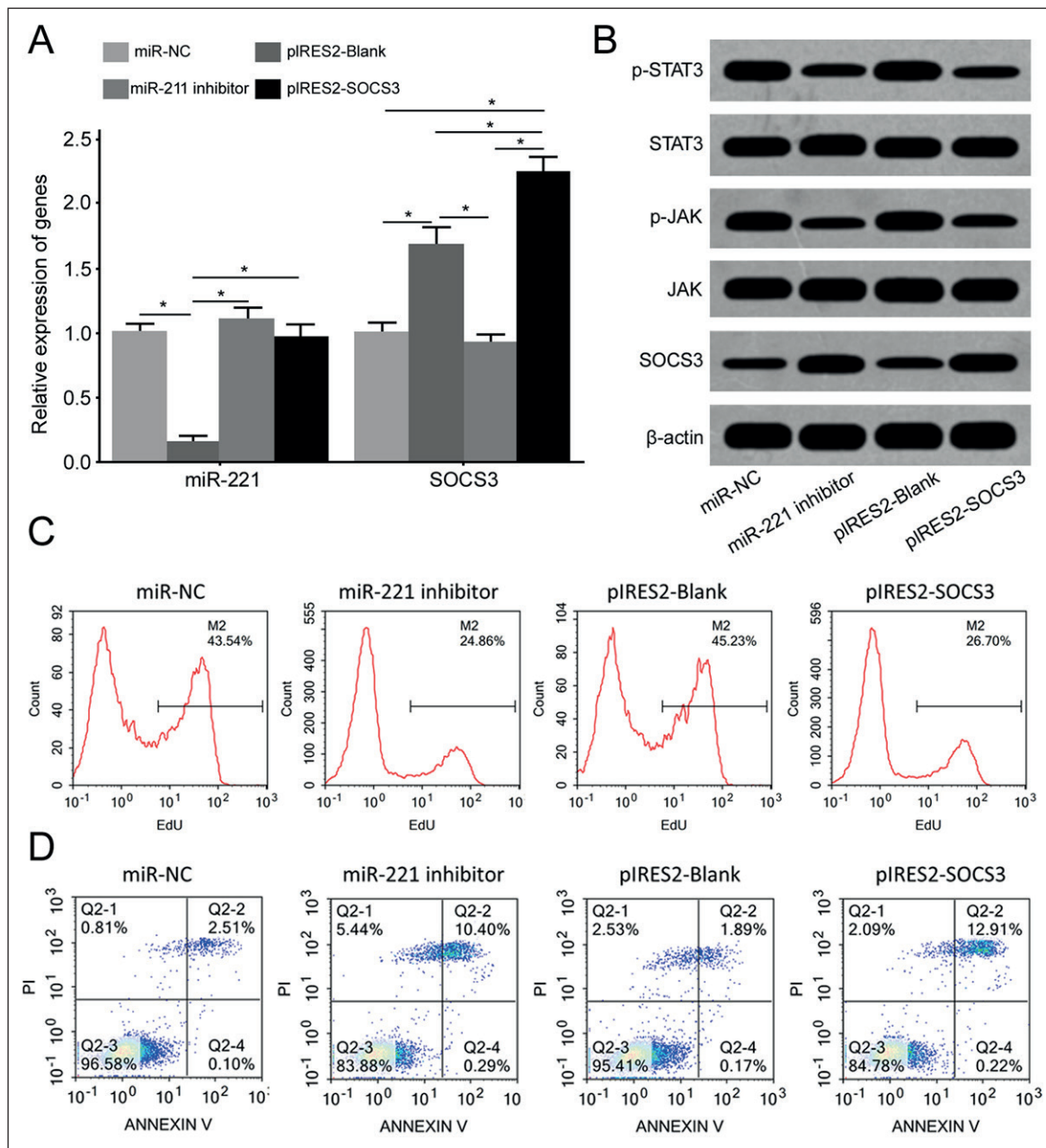


Figure 3. MiR-221 down-regulation suppressed PC cell proliferation and induced apoptosis. **A**, qRT-PCR for gene expression; **B**, Western blot for protein expression; **C**, EdU staining for cell proliferation; **D**, Flow cytometry for cell apoptosis. *, $p < 0.05$ comparing between two groups.

to normal pancreatic ductal epithelium, PC cells showed remarkably elevated miR-221 expression. They also found that compared to tumor adjacent tissues, PC tissues showed abnormally elevated miR-221 expression, and those up-regulated serum miR-221 were correlated with patient TNM stage and distal metastasis, plus relatively higher value for differential diagnosis of distal metastatic PC (AUC=0.689). Xu et al²⁵ demonstrated that compa-

red to non-infiltrative PC cell line Bxpc-3, infiltrative PC cell line SW-1990, PANC-1 and Miapaca-2 showed remarkably elevated miR-221 expression. Similarly, we observed that compared to normal pancreatic epithelium, miR-221 expression was abnormally elevated in PC cells showed, indicating its potential oncogene role in PC pathogenesis, which was in line with previous finding that compared to normal pancreatic tissues. PC tumor

tissues also showed remarkably elevated miR-221 expression, which was inversely correlated with patient survival²⁶.

Of note, our data on dual luciferase gene reporter assay confirmed the targeted regulatory relationship between miR-221 and SOCS3. The expression of SOCS3 and G0/G1 phase arresting were significantly decreased. We proposed that miR-221 up-regulation might play a role in suppressing SOCS3 expression, enhancing JAK-STAT3 pathway activity, and facilitating PC cell proliferation and cycle progression. Further study elucidated that the suppression of miR-221 remarkably up-regulated SOCS3 expression in PANC-1 cells, which also impeded the cell proliferation. Sarkar et al²⁶ found that after using drugs such as G2535, BR-DIM or CDF to suppress miR-221 expression, the expression of tumor suppressor genes including PTEN, p27 (kip1), p57 (kip2) and PUMA was significantly elevated in PC cells such as MiaPaCa-2 or PANC1, whilst cell proliferation or migration potency was compromised. Tanaka et al²⁷ found abnormally elevated miR-221 expression in PC cells. After metformin drug treatment, the expression of target gene p27Kip1 was remarkably elevated, accompanied with more G1 phase arresting and weakened proliferation of PC cells, which was in agreement with our study. Furthermore, Lesina et al²⁸ found that knockdown of SOCS3 expression remarkably up-regulated STAT3 transcriptional activity, thus facilitating progression of PC. Patel²⁹ showed that SOCS3 down-regulation was correlated with malignant biological properties of PC cells. When SOCS3 expression was enhanced, activity of JAK kinase and STAT3 were remarkably weakened, and growth or migration potency of PC cells was significantly restricted. This investigation observed the correlation between STAT3 downregulation and malignant biological behaviors of PC cells, and SOCS3 up-regulation significantly weakened PC malignant features. However, whether such modulatory relationship existed between miR-221 and SOCS3 in PC patients *in vivo* still remains unclear, and further investigations are thus required to elucidate expressional profiles of miR-221 and SOCS3 in PC tissues.

Conclusions

We found that miR-221 inhibits SOCS3 expression. Down-regulation of miR-221 enhances SOCS3 expression, suppresses activity of JAK-STAT3 pathway, remarkably weakening proliferation potency of PC cell line PANC-1.

Acknowledgments:

This work was supported by the Precancerous radiotherapy of pancreatic cancer related research (2016ZC100).

Conflict of Interest:

The Authors declare that they have no conflict of interests.

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