Inhibition of miR-221 influences bladder cancer cell proliferation and apoptosis

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Abstract. – OBJECTIVE: Janus kinase (JAK) - signal transducer and activator of transcription (STAT) signaling pathway participate in cell proliferation and apoptosis. Suppressors of cytokine signaling 3 (SOCS3) are negative regulators of JAK-STAT3. SOCS3 was found significantly declined, while microRNA-221 (miR-221) obviously up-regulated in bladder cancer tissue. Bioinformatics analysis revealed the complementary binding site between miR-221 and 3'-UTR of SOCS3. This study investigated the role of miR-221 in regulating SOCS3/JAK-STAT3 signaling pathway and bladder cancer cell proliferation and apoptosis.

PATIENTS AND METHODS: Bladder cancer tumor tissue and para-carcinoma tissue were collected from patients to test miR-221 and SOCS3 expressions. Dual luciferase assay was used to test the targeting regulatory effect of miR-221 on SOCS3. MiR-221, SOCS3, p-JAK1, p-JAK2, and survivin expressions were compared in T24 and HBEC cells. T24 cells were divided into miR-NC, miR-221 inhibitor, pSicoR-blank, pSicoR-SOCS3, and miR-221 inhibitor + pSicoR-SOCS3 groups. Flow cytometry was applied to detect cell apoptosis, EdU staining was adopted to evaluate cell proliferation.

RESULTS: MiR-221 significantly increased, while SOCS3 obviously reduced in bladder cancer tissue compared with para-carcinoma tissue. MiR-221 targeted inhibited SOCS3 expression. MiR-221, phosphorylated JAK1 (p-JAK1), phosphorylated JAK2 (p-JAK2), phosphorylated STAT3 (p-STAT3), and survivin levels markedly up-regulated, whereas SOCS3 expression apparently declined in T24 cells compared with that in HBEC cells. MiR-221 inhibitor and/or pSicoR-SOCS3 elevated SOCS3 expression, decreased p-JAK1, p-JAK2, p-STAT3, and survivin levels, enhanced cell apoptosis, and attenuated cell proliferation.

CONCLUSIONS: MiR-221 elevated, while SOCS3 reduced in bladder cancer tissue. Inhibition of miR-221 suppressed T24 cell proliferation and induced apoptosis by up-regulating SOCS3 expression, lowering JAK-STAT3 signaling pathway activity, and attenuating survivin expression.

Key Words: miR-221, SOCS3, JAK-STAT3, Bladder cancer, Proliferation, Apoptosis.
miRNA expression and function in tumorigenesis receive more and more attention. MiR-221 was shown significantly up-regulated in BC tissue, suggesting its potential oncogene role in BC occurrence. Bioinformatics analysis revealed the complementary binding site between miR-221 and 3'-UTR of SOCS3. This study investigated the role of miR-221 in regulating SOCS3/JAK-STAT3 signaling pathway and bladder cancer cell proliferation and apoptosis.

Patients and Methods

Patients
A total of 42 BC patients were enrolled, including 32 males and 10 females with mean age at 62.9 (51-78) years old. Patients received treatment in Huaihe Hospital, Henan University between Apr and Nov 2016. Tumor tissue and para-carcinoma tissue were collected and stored at -80°C for RNA, protein, and immunofluorescence detection.

This study was approved by Ethics Committee in Huaihe Hospital, Henan University and all the enrolled objects had signed informed consent.

Main Reagents and Materials

Human BC cell T24 was purchased from Suer (Shanghai, China). Human normal bladder epithelial cell HBEC was bought from Yubo Biological Technology Co., Ltd. (Shanghai, China). Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). EasyPure RNA Kit was obtained from TransGen Biotech (Beijing, China). Transfection reagent Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). PrimerScript™ RT reagent Kit and SYBR Green were purchased from Takara (Dalian, China). miR-NC, miR-221 mimic, miR-221 inhibitor, and EdU cell proliferation detection kit were purchased from Ribobio (Guangzhou, China). Mouse anti-human p-JAK1, p-JAK2, and SOCS3 primary antibodies were purchased from Abcam Biotechnology (Cambridge, MA, USA). Rabbit anti-human p-STAT3 and survivin primary antibodies were obtained from GenTex Inc. (Irvine, CA, USA). Horseradish Peroxidase (HRP) conjugated secondary antibody was derived from Bio-Rad Laboratories (Hercules, CA, USA). Fluorescence secondary antibody was purchased from Molecular Probes (Eugene, OR, USA). pGRE-luc reporter gene plasmid, dual luciferase detection kit, apoptosis detection kit, and radio-immunoprecipitation assay (RIPA) were bought from Beyotime (Shanghai, China). pSicoR-GFP was obtained from Addgene (London, UK).

Cell Culture
T24 and HBEC cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS and 1% penicillin-streptomycin. Cells were passaged at 1:4.

Dual-Luciferase Reporter Gene Assay
The polymerase chain reaction (PCR) products containing the full length of SOCS3 gene 3'-UTR or mutant segment were cloned to pGRE-luc. Next, it was transformed to DH5α competent cells and sequenced to select the plasmid with correct sequence, namely pGRE-SOCS3-wt and pGRE-SOCS3-mut, respectively. Then, pGRE-SOCS3-wt (or pGRE-SOCS3-mut) was co-transfected to HEK293T cells by using Lipofectamine 2000, together with miR-221 mimic (or miR-221 inhibitor, or miR-NC). The luciferase activity was detected after cultured for 48 h.

SOCS3 Over-Expression Plasmid Construction
CDS segment of SOCS3 gene was amplified and connected to pSicoR-GFP plasmid after double digestion. After transformation, the positive bacterial strain was selected and amplified to extract the recombinant plasmid. The plasmid with correct sequence was named pSicoR-GFP-SOCS3. pSicoR-GFP-blank was treated as control.

Cell Transfection and Grouping
T24 cells were divided into miR-NC, miR-221 inhibitor, pSicoR-blank, pSicoR-SOCS3, and miR-221 inhibitor + pSicoR-SOCS3 groups. Cells were detected on 72 h after transfection.

qRT-PCR
Total RNA was extracted by using EasyPure RNA Kit and reverse transcribed to cDNA by using PrimerScript™ RT reagent Kit. The reverse transcription condition was 37°C for 15 min and 98°C for 5 min. The PCR reaction was composed of 95°C pre-denaturation for 5 min, followed by 40 cycles of 95°C denaturation for 15 s, 60°C annealing for 30 s, and 74°C elongation for 30 s. Real-time PCR was performed on CFX96 Touch™ to test the relative expression. The primers used were as follows. miR-221P.
Inhibition of miR-221 influences bladder cancer cell proliferation and apoptosis

5'-GTTGGTGGGAGCTACATTGTCTGC-3', miR-221P; 5'-GTGTCGTGGACCTGGACATT-3'; U6P F: 5'-GGAACGCCGTCAAGACGCAATT-3'; SOC-S3P F: 5'-CGCTGACCTGCGCTCAAC-3', SOC-S3P R: 5'-GTCACTGCGCTTCAGTAGA-3'; SurvivinP F: 5'-AGGACCACCAGCTCAGATCAT-3', SurvivinP R: 5'-AGAATGGCTCGTTCTCA-3', β-actinP F: 5'-GAACCCTTACAAC-3', β-actinP R: 5'-TGTCACGCACGATTTCC-3'.

Western Blot
Total protein was extracted by RIPA from tissue and cells. A total of 50 μg protein was separated by 10% sodium-lauryl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked and incubated with primary antibody at 4°C overnight (SOCS3, p-JAK1, p-JAK2, p-STAT3, survivin, and β-actin at 1:300, 1:200, 1:200, 1:200, and 1:800, respectively). Then the membrane was incubated with secondary antibody (1:10000) for 60 min after washed by phosphate buffer saline-tween 20 (PBST) for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

Flow Cytometry
The cells were re-suspended in 500 μl binding buffer and incubated with 5 μl Annexin V-FITC avoid of light for 15 min. Next, cells were added to 5 μl PI and tested on EPICS XL-MCL flow cytometry to evaluate cell apoptosis.

EdU Staining
Cells were added to EdU solution in logarithmic phase and were incubated for 48 h. Then, cells were digested by 0.125% trypsin and were collected; after, they were fixed in paraformaldehyde and neutralized in glycine. Next, cells were incubated with 0.1% Triton X-100 and re-suspended in PBS. At last, the cells were stained by 500 μl Apollo at room temperature for 10 min and tested on EPICS XL-MCL flow cytometry (BD Biosciences, San Jose, CA, USA).

Immunofluorescence Detection
The frozen section was fixed in precooled acetone at room temperature for 10 min. After the section was washed by phosphate buffer saline (PBS) for three times, it was blocked in PBS containing 2% bovine serum albumin (BSA) and 0.1% Triton X-100 at room temperature for 60 min. Next, the section was incubated with mouse anti-human SOCS3 primary antibody at 4°C overnight. Then, the section was incubated with Alexa Fluor 594 labeled secondary antibody at room temperature for 60 min. At last, the section was stained by 0.1% 4',6-diamidino-2-phenylinodole (DAPI) for 1 min and observed under the microscope.

Statistical Analysis
All data analyses were performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by t-test or Mann-Whitney U test. *p<0.05 was considered as statistical significance.

Results

**MiR-221 upregulated, while SOCS3 Reduced in BC tissue**
qRT-PCR detection revealed that miR-221 expression was significantly higher in cancer tissue than in para-carcinoma tissue (Mann-Whitney U value = 5.000, p<0.001) (Figure 1A). Immunofluorescence detection showed that SOCS3 level obviously reduced in tumor tissue compared with adjacent normal control (Figure 1B).

![Figure 1](image-url)
MiR-221 Targeted regulated SOCS3 Expression in T24 Cells

MiR-221 expression was obviously higher, while SOCS3 level was markedly lower in T24 cells compared with HBEC cells, suggesting the potential regulatory relationship between miR-221 and SOCS3 (Figure 2A and B). Western blot demonstrated that p-JAK1, p-JAK2, p-STAT3, and survivin levels markedly up-regulated in T24 cells compared with that in HBEC cells (Figure 2B). microRNA.org online prediction showed the complementary binding site between miR-221 and the 3′-UTR of SOCS3 mRNA (Figure 2C). Dual luciferase assay revealed that miR-221 mimic and miR-221 inhibitor significantly declined and enhanced relative luciferase activity in HEK293 cells (Figure 2D), indicating the regulatory relationship between miR-221 and SOCS3.
mRNA. MiR-221 mimic or inhibitor transfection apparently reduced or elevated SOCS3 mRNA expression in T24 cells, confirming that miR-221 targeted regulated SOCS3 expression (Figure 2E).

**Inhibition of miR-221 Upregulated SOCS3 expression, Suppressed T24 cell Proliferation, and Promoted Apoptosis**

MiR-221 inhibitor and/or pSicoR-SOCS3 elevated SOCS3 expression, decreased p-JAK1, p-JAK2, p-STAT3, and survivin levels (Figure 3A and B), enhanced cell apoptosis (Figure 3C), and attenuated cell proliferation (Figure 3D).

**Discussion**

JAK-STAT signaling pathway could be activated by various extra-cellular cytokines, growth factors, and mitogen, leading to receptor dimerization, which phosphorylated JAK kinase. Activated JAK phosphorylates membrane receptor, leading to conformation, changes to promote STAT, covering the tyrosine phosphorylation loci on membrane receptor complex. At this time, JAK kinase phosphorylates STAT to let it form dimer with another STAT, thus entering the nucleus to regulate gene transcription and expression. STAT transcription

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**Figure 3.** Inhibition of miR-221 up-regulated SOCS3 expression, suppressed T24 cell proliferation, and promoted apoptosis. (A) qRT-PCR detection of miR-221 and SOCS3 expressions; (B) Western blot detection of protein expressions; (C) flow cytometry detection of cell apoptosis; (D) EdU staining detection of cell proliferation. *p*<0.05, compared with miR-NC; *p*<0.05, compared with pSicoR-blank; *p*<0.05, compared with miR-221 inhibitor; *p*<0.05, compared with pSicoR-SOCS3.
factor family contains seven members, including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. STAT3 is most widely investigated and exhibits the closest relationship with tumor occurrence and development. Inhibitor of apoptosis protein (IAPs) is a protein family with homological structure and anti-apoptosis function. Survivin is the most important member of IAPs family with the strongest function in apoptosis inhibition. Survivin blocks cell apoptosis by inhibiting caspase-3 and caspase-7. Moreover, survivin also plays a crucial role in regulating cell cycle and facilitating cell proliferation. It was showed that JAK-STAT3 signaling pathway is involved in decreasing SOCS3 expression and promoting malignancy and development, including colorectal cancer, lung cancer, and breast cancer. Recently it was found that SOCS3 expression abnormally reduced in bladder cancer tissue, indicating its promoting role in bladder cancer tumorigenesis. Gottardo et al demonstrated that miR-221 elevation plays a role in suppressing T24 cell proliferation and induced apoptosis by up-regulating SOCS3 expression, lowering JAK-STAT3 signaling pathway activity, and attenuating survivin expression.

Conclusions

MiR-221 elevated, while SOCS3 reduced in bladder cancer tissue. Inhibition of miR-221 suppressed T24 cell proliferation and induced apoptosis by up-regulating SOCS3 expression, lowering JAK-STAT3 signaling pathway activity, and attenuating survivin expression.
Inhibition of miR-221 influences bladder cancer cell proliferation and apoptosis

Conflict of Interest
The Authors declare that they have no conflict of interests.

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4) Rose TL, Deal AM, Nielsen ME, Smith AB, Miesgal. Prognostic value of

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