**SOCS3 overexpression enhances ADM resistance in bladder cancer T24 cells**

M.-Z. Li¹, D.-H. Lai², H.-B. Zhao², Z. Chen¹, Q.-X. Huang¹, J. Situ¹

¹Department of Urology, The Third Affiliated Hospital Sun Yat-Sen University, Guangzhou, Guangdong, China
²Department of Urology, Fifth Affiliated Hospital Guangzhou Medical University, Guangzhou, Guangdong, China

**Abstract.** – **OBJECTIVE:** JAK-STAT3 signaling pathway widely participates in cell proliferation and apoptosis. Suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of JAK-STAT3. SOCS3 downregulation is associated with drug resistance in breast cancer and leukemia. However, its role in bladder cancer drug resistance is still unclear. This study established ADM resistant bladder cancer cell model to investigate the role of SOCS3-JAK/STAT3 signaling pathway ADM resistance.

**MATERIALS AND METHODS:** ADM drug resistant cell line T24/ADM was established. SOCS3, p-JAK2, p-JAK3, and Bcl-2 expressions in T24/ADM, T24, and HBEC cells were compared. Cell proliferation and apoptosis were evaluated by flow cytometry. T24/ADM cells were divided into five groups, including control, pSicoR-blank, pSicoR-SOCS3, FLLL32, and pSicoR-SOCS3 + FLLL32 groups. Cell proliferation was determined by EdU staining.

**RESULTS:** SOCS3 was reduced, while p-JAK2, p-JAK3, and Bcl-2 expressions upregulated in T24 cells compared with HBEC cells. T24/ADM cells exhibited lower SOCS3, higher p-JAK2, p-JAK3, and Bcl-2 levels than T24 cells. Cell apoptosis was higher, whereas cell proliferation was weaker in T24 cells compared with T24/ADM cells. SOCS3 overexpression and/or FLLL32 treatment significantly downregulated p-JAK2, p-JAK3, and Bcl-2 expressions, attenuated cell proliferation, and elevated sensitivity to ADM induced cell apoptosis.

**CONCLUSIONS:** SOCS3 reduction was associated with bladder cancer sensitivity to ADM. SOCS3 overexpression decreased JAK-STAT3 signaling pathway activity, declined Bcl-2 expression, inhibited cell proliferation, elevated cell apoptosis, and enhanced ADM sensitivity in T24 cells.

**Key Words:** SOCS3, JAK-STAT3, T24, ADM, Drug resistance.

**Introduction**

As the most common urinary system tumor, bladder cancer exhibits the leading morbidity and mortality in China. Chemotherapy is an important method for its postoperative treatment. However, some patients in advanced stage show poor sensitivity to chemotherapy, leading to poor therapeutic effect. Adriamycin (ADM) is the first line drug for bladder cancer. Long-term application of ADM may cause drug resistance in bladder cancer. Therefore, exploring the mechanism of bladder cancer drug resistance is of great significance to decrease drug resistance, elevate chemotherapy effect, and improve the prognosis of bladder cancer. Multiple mechanisms are involved in mediating chemotherapy resistance, such as cell survival, proliferation, and apoptosis. Regulation of cell survival, proliferation, and apoptosis may change the sensitivity to chemotherapy drugs. Janus kinase (JAK) - signal transducer and activator of transcription (STAT) signaling pathway widely exists in various tissues and cells. It participates in the regulation of cell proliferation, cycle, and apoptosis. It was showed that JAK-STAT3 activity enhancement was related to drug resistance in lung cancer, leukemia, and lymphoma. Suppressor of cytokine signaling 3 (SOCS3) is a member of SOCS family with the strongest activity. It suppresses JAK-STAT signaling pathway activation and transduction. It was revealed that SOCS3 downregulation was associated with drug sensitivity reduction in breast cancer, suggesting its potential role in alleviating cancer cell drug resistance. However, its impact in bladder cancer drug resistance is still unclear. This study established ADM resistant bladder cancer cell model to investigate the role of SOCS3-JAK/STAT3 signaling pathway ADM resistance.

**Corresponding Author:** Jie Situ, MD; e-mail: jiesitu668@163.com
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**Materials and Methods**

**Main Reagents and Materials**

Human bladder cancer cell T24 and normal bladder epithelial cell HBEC were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Dulbecco’s Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were got from Gibco (Rockville, MD, USA). Trizol was bought from Invitrogen (Carlsbad, CA, USA). ReverTra Ace qPCR RT Kit and SYBR were obtained from Toyobo (Tokyo, Japan). Rabbit anti-human SOCS3 and p-JAK2 primary antibodies were purchased from Abcam (Cambridge, MA, USA). Mouse anti-human p-STAT3 and Bcl-2 primary antibodies were purchased from CST (Danvers, MA, USA). Mouse anti-human β-actin primary antibody was got from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP conjugated secondary antibody was bought from Bio-Rad (Hercules, CA, USA). ADM was derived from Hengrui Medicine (Jiangsu, China). EdU staining kit was obtained from RiboBio (Guangzhou, China). Annexin V-FITC/PI cell apoptosis detection kit, caspase-3 activity detection kit, and RIPA were purchased from Beyotime (Jiangsu, China). JAK/STAT3 inhibitor FLLL32 was bought from Selleck (Houston, TX, USA). pSicoR-GFP was obtained from Addgene (Cambridge, MA, USA). CCK-8 reagent was derived from Dojindo (Kumamoto, Japan).

**Cell Culture**

T24 and HBEC cells were cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin. Cells were passaged at 1:4.

**T24/ADM Drug Resistant Cell Line Establishment**

T24 cells in logarithmic phase were treated by ADM from 0.2 mg/L for 24 h. Then cells kept growing and were treated by increased concentration of ADM up to 2.0 mg/L when the cells can stable grow in ADM. At last, cells can be stable passaged in ADM to obtain ADM resistant bladder cancer cell line T24/ADM. T24 and T24/ADM cells were treated by different concentrations of ADM for 48 h. Next, cells were added with CCK-8 to measure the absorbance value (A450). Inhibition rate = (1-A450 in drug group)/A450 in control × 100%. IC50 was calculated using SPSS18.0 software (SPSS Inc., Chicago, IL, USA). Resistance index (RI) = IC50 of drug resistant cell/IC50 of parent cell.

**Cell Treatment and Grouping**

T24/ADM cells were divided into five groups, including control, pSicoR-blank, pSicoR-SOCS3, FLLL32 (1.0 μM), and pSicoR-SOCS3 + FLLL32 (1.0 μM) groups. Cells were treated by ADM at 2.0 mg/L for 72 h before following experiments.

**qRT-PCR**

Total RNA was extracted using Trizol and reverse transcribed to cDNA using ReverTra Ace qPCR RT Kit. The PCR reaction was performed under the effect of TaqDNA polymerase. The primers used were as follows.

- SOCS3F: 5′-CCTGCGCCTCAAGACCTTC-3′
- SOCS3R: 5′-GTCACTGCGCTCCAGTAGAA-3′
- Bcl-2F: 5′-GGTGGGGTCATGTGTGTGG-3′
- Bcl-2R: 5′-CGGTTCAGGTACTCAGTCATCC-3′
- β-actinF: 5′-GAACCCTAAGGCCAAC-3′
- β-actinR: 5′-TGTCACGCACGATTTCC-3′

**Western Blot**

Total protein was extracted by radioimmunoprecipitation assay (RIPA) on ice for 30 min. After centrifuged at 12000 rpm for 10 min, a total of 50 μg protein was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked by 5% skim milk and was incubated in primary antibody at 4°C overnight (SOCS3, p-JAK2, p-STAT3, Bcl-2, and β-actin at 1:400, 1:200, 1:200, and 1:600, respectively). Then the membrane was incubated in secondary antibody (1:8000) for 60 min and then it was washed by PBST for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).

**EdU Staining**

Cells were added to EdU solution in logarithmic phase. After incubated for 48 h, cells were digested by 0.125% trypsin and collected. After they were fixed in paraformaldehyde, and were neutralized in glycine. Next, cells were blocked by 5% skim milk and was incubated in primary antibody at 4°C overnight (SOCS3, p-JAK2, p-STAT3, Bcl-2, and β-actin at 1:400, 1:200, 1:200, and 1:600, respectively). Then the membrane was incubated in secondary antibody (1:8000) for 60 min and then it was washed by PBST for three times. At last, the protein expression was detected by enhanced chemiluminescence (ECL).
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Flow Cytometry
Cells were re-suspended in 500 μl binding buffer and were incubated in 5 μl Annexin V-FITC and 5 μl PI. Next, cells were tested on EPICS XL-MCL flow cytometry to evaluate cell apoptosis.

Caspase-3 Activity Detection
According to the manual, pNA standard substance was established and tested at A405 to draw the standard curve. Cells were digested by trypsin and cracked on ice. Then the 96-well plate was incubated with buffer, sample, Ac-DEVD-pNA at 37°C for 2 h. A405 was tested to reflect caspase-3 activity.

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-tests. \( p < 0.05 \) was considered as statistical significance.

Results
IC50 Increased in T24/ADM Cells
SPSS software revealed that IC50 of T24 cells was 0.69 mg/L, while it was 7.82 mg/L in T24/ADM cells. The RI of T24/ADM was 11.33. Caspase-3 activity (Figure 1 A) and cell apoptosis (Figure 1 B) were higher, while cell proliferation (Figure 1 C) was significantly lower in T24 cells compared with T24/ADM cells after treated by ADM with concentration at T24 IC50.

SOCS3 Level Reduction, JAK-STAT3 Activity and Bcl-2 Expression Elevation were Related to T24 drug Resistance
qRT-PCR demonstrated that SOCS3 mRNA markedly declined, while Bcl-2 mRNA apparently enhanced in T24 cells compared with HBEC cells. T24/ADM cells exhibited lower SOCS3 and higher Bcl-2 levels than T24 cells (Figure 2 A). Western blot showed that SOCS3 protein significantly decreased, whereas p-JAK2, p-STAT3, and Bcl-2 proteins markedly upregulated in T24 cells compared with HBEC cells. T24/ADM cells revealed lower SOCS3 protein, and higher p-JAK2, p-STAT3, and Bcl-2 protein levels than T24 cells (Figure 2 B).

SOCS3 Upregulation Declined T24/ADM Drug Resistance
SOCS3 mRNA and protein levels significantly increased in pSicoR-SOCS3 transfection group compared with pSicoR-blank group, suggesting successful SOCS3 overexpression in T24/ADM cells (Figure 3 A-B). SOCS3 mRNA and protein were elevated, while p-JAK2, p-STAT3, and Bcl-2 protein levels markedly declined in T24/ADM cells transfection by pSicoR-SOCS3 compared with control (Figure 3 C-D). Caspase-3 activity (Figure 3 E) and cell apoptosis (Figure 3 F) apparently increased, while cell proliferation (Figure 3 G) significantly attenuated in T24/ADM cells treated by 2.0 mg/L ADM. FLLL32 treatment at 1.0 μM also downregulated p-JAK2, p-STAT3, and Bcl-2 expressions, inhibited cell prolifera-
tion, and promoted cell apoptosis in T24/ADM cells. pSicoR-SOCS3 transfection combined FLLL32 treatment exhibited the strongest impact.

Discussion

B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic factor depending on mitochondrial pathway. It plays an anti-apoptotic effect through multiple mechanisms, such as changing mitochondrial membrane permeability and inhibiting cytochrome C release. Bcl-2 upregulation is closely related to cell apoptosis reduction and drug resistance in various cancer cells, such as cervical cancer, gastric cancer, and lung cancer. JAK-STAT signaling pathway is found to play an important role in cell proliferation, cycle, and apoptosis. Activated JAK phosphorlatorylates membrane receptor, leading to conformation, changes to promote STAT, covering the tyrosine phosphorylation loci on membrane receptor complex. At this time, JAK kinase phosphorlates STAT to let it form dimer with another STAT, thus entering the nucleus to regulate gene transcription and expression. Numerous studies demonstrated that Bcl-2 is one of the important target genes of JAK-STAT3 signaling pathway. JAK-STAT3 mediated Bcl-2 upregulation and cell apoptosis reduction are associated with drug resistance in cancer cells, including lung cancer and colorectal cancer. SOCS3 is the strongest member in SOCS family that can inhibit JAK phosphorylation and the activity of JAK kinase. It is a critical negative regulator of JAK-STAT3. It was showed that SOCS3 decrease was related to chemotherapeutic drug sensitivity reduction in breast cancer cells, indicating that SOCS3 may have the effect of lowering drug resistance. However, its role in bladder cancer drug resistance is still unclear. This study established ADM resistant bladder cancer cell model to investigate the role of SOCS3-JAK/STAT3 signaling pathway ADM resistance. We revealed that IC50 in T24/ADM cells was significantly higher than that in T24 cells. Cell apoptosis was higher, while cell proliferation was significantly lower in T24 cells compared with T24/ADM cells. It suggested the successful establishment of ADM resistant T24/ADM cells. SOCS3 reduced, while p-JAK2, p-STAT3, and Bcl-2 expressions upregulated in T24 cells compared with HBEC cells. It indicated that SOCS3 downregulation mediated JAK-STAT3 activity enhancement and Bcl-2 elevation were associated with bladder cancer occurrence. Gaballah et al. revealed that SOCS3 expression markedly declined in bladder cancer tissue compared with normal control. Its expression was related to pathological grading, as it declined in patients with higher pathological grade. We demonstrated that SOCS3 expression in bladder cancer cells was markedly lower than that in normal bladder epithelial cells, revealing that SOCS3 downregulation was related to bladder cancer occurrence, which was similar to Gabal-
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Moreover, T24/ADM cells revealed lower SOCS3 protein, higher p-JAK2, p-STAT3, and Bcl-2 protein levels than T24 cells. It showed that SOCS3 downregulation mediated JAK-STAT3 activity enhancement and Bcl-2 elevation may play a regulatory role in bladder cancer drug resistance. Souckova et al. reported that SOCS3 lower expression and dysfunction was related to chemotherapeutic drug sensitivity decrease in breast cancer cells. Liu et al. revealed that SOCS3 expression was closely associated with leukemia cell K562 and KU812 sensitivity to imatinib. However, the relation between SOCS3 expression and drug resistance in bladder cancer needs further study.

**Figure 3.** SOCS3 upregulation declined T24/ADM drug resistance. (A) qRT-PCR detection of SOCS3 mRNA expression in T24/ADM cells; (B) Western blot detection of SOCS3 protein expression in T24/ADM cells; (C) qRT-PCR detection of mRNA expression in T24/ADM cells; (D) Western blot detection of protein expression in T24/ADM cells; (E) Spectrophotometry detection of caspase-3 activity; (F) Flow cytometry detection of cell apoptosis; (G) Flow cytometry detection of cell proliferation. (a) $p < 0.05$, compared with pSicoR-blank; (b) $p < 0.05$, compared with control; (c) $p < 0.05$, compared with pSicoR-SOCS3; (d) $p < 0.05$, compared with FLLL32.
bladder cancer cell drug resistance was still unreported. Our results indicated that SOCS3 downregulation may play a promoting role in bladder cancer cell ADM resistance. Thus, this work further explored the regulatory role of SOCS3-JAK-STAT3 in bladder cancer cell ADM resistance through intervening SOCS3 expression and downstream JAK-STAT3 pathway activity. SOCS3 overexpression and/or FLLL32 treatment significantly downregulated Bcl-2 expressions, attenuated cell proliferation, and elevated sensitivity to ADM induced cell apoptosis. Yang et al. reported that JAK-STAT3 signaling pathway activation marked markedly enhanced bladder cancer cell migration and invasion. Zhang et al. showed that reducing STAT3 expression and function inhibit bladder cancer T24 cell proliferation and survival. Bednarek et al. revealed that reducing STAT3 may attenuate T24 cell proliferation and accelerate cell apoptosis. Joung et al. demonstrated that JAK2 kinase inhibitor AG490 markedly declined JAK-STAT3 signaling pathway, weakened cell proliferation and migration, and restrained cell growth in in vivo. In this study, SOCS3 overexpression and/or JAK-STAT3 signaling pathway blockade significantly weakened bladder cancer cell malignancy, which was similar to Zhang et al. and Joung et al. discovered that JAK-STAT3 signaling pathway enhancement facilitated autophagy to strengthen cisplatin resistance in bladder cancer cells. On the contrary, inhibiting JAK kinase activity obviously declined stemness gene and drug resistance gene expressions, restrained cell proliferation, and enhanced cisplatin sensitivity. This study confirmed that JAK-STAT3 signaling pathway plays a regulatory role in T24 cell ADM resistance, which was in accordance with Ojha et al. We also revealed that SOCS3 downregulation plays a regulatory role in enhancing JAK-STAT3 signaling pathway and drug resistance in bladder cancer cells.

Conclusions

SOCS3 reduction was associated with bladder cancer sensitivity to ADM. SOCS3 overexpression decreased JAK-STAT3 signaling pathway activity, declined Bcl-2 expression, inhibited cell proliferation, elevated cell apoptosis, and enhanced ADM sensitivity in T24 cells.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

References


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