Int J Clin Exp Med 2019;12(10):12228-12235
www.ijcem.com /ISSN:1940-5901/IJCEM0095083

Original Article

MiR-221 affects cholangiocarcinoma cell proliferation and apoptosis by targeting the SOCS3/JAK-STAT signaling pathway

Yugang Cao[1], Guobing Xia[1], Jun Guo[1], Kai Cheng[2]

[1]Department of Hepatobiliary Surgery, [2]Gastrointestinal Surgery, Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Edong Healthcare Group, Huangshi 435000, Hubei, China

Received April 9, 2019; Accepted July 10, 2019; Epub October 15, 2019; Published October 30, 2019

Abstract: Cytokine-mediated tyrosine kinase (JAK)-signal transducer and transcriptional activator (STAT) pathway activation is associated with tumorigenesis. Cytokine signal transduction inhibitor 3 (SOCS3) negatively regulates JAK-STAT, and its decreased expression is associated with a variety of tumors. MiR-221 expression was significantly elevated in cholangiocarcinoma tissues. Bioinformatics analysis revealed a targeted binding site between miR-221 and the 3'-UTR of SOCS3 mRNA. The present study assessed the role of miR-221 in regulating SOCS3 expression and affecting cholangiocarcinoma cell proliferation and apoptosis. The target interaction between miR-221 and SOCS3 was validated by dual luciferase reporter gene assay. Human normal biliary cells HIBEC, cholangiocarcinoma QBC939 and HUCCT1 cells were cultured *in vitro*. The expressions of miR-221 and SOCS3 mRNA were detected by qRT-PCR. SOCS3 protein expression was detected by western blot. QBC939 and HUCCT1 cells were divided into miR-NC group and miR-221 inhibitor group. Cell apoptosis was detected by flow cytometry. Cell proliferation was detected by EdU staining. There is a targeted regulation relationship between miR-221 and SOCS3. Compared with HIBEC cells, miR-221 expression was significantly increased, while SOCS3 expression clearly declined in cholangiocarcinoma QBC939 and HUCCT1 cells. MiR-221 inhibitor transfection markedly upregulated SOCS3 expression, suppressed JAK-STAT pathway activity, increased cell apoptosis, and attenuated cell proliferation in cholangiocarcinoma QBC939 and HUCCT1 cells. Increased miR-221 expression and decreased SOCS3 level are associated with the pathogenesis of cholangiocarcinoma. MiR-221 can regulate the proliferation and apoptosis of cholangiocarcinoma cells by specifically inhibiting SOCS3 expression and affecting JAK-STAT pathway activity.

Keywords: miR-221, SOCS3, JAK-STAT, cholangiocarcinoma

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor originating from intrahepatic and extrahepatic bile duct epithelial cells, accounting for 30% of biliary malignant tumors and 3% of all digestive tract malignancies [1, 2]. CCA is a kind of malignant tumor. The early stage of the disease is relatively insidious and does not cause typical symptoms. However, it shows strong invasive and metastasis ability. Most patients suffer from adjacent tissue infiltration or distant metastasis when diagnosed, thus the prognosis is poor [3, 4].

The tyrosine kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway participates in regulating various biological processes, such as cell survival, proliferation, cycle, apoptosis, migration and invasion [5, 6]. Suppressors of cytokine signaling 3 (SOCS3) can inhibit JAK kinase activity and phosphorylation of STAT, thus negatively regulating JAK-STAT signaling [7]. SOCS3 is associated with the occurrence, progression, and prognosis of various tumors [8-10]. Abnormal expression of SOCS3 is associated with the occurrence, progression and drug resistance of cholangiocarcinoma [11, 12].

MiR-221 is a well-studied microRNA, which is related to the occurrence, progression and metastasis of various tumors [13-15]. A number of studies revealed that abnormal expression of

miR-221 is associated with the occurrence and development of cholangiocarcinoma, suggesting that miR-221 may be a cancer-promoting factor in the pathogenesis of cholangiocarcinoma [16-18]. Bioinformatics analysis exhibited a targeted binding site between miR-221 and the 3'-UTR of SOCS3 mRNA. This study evaluated the role of miR-221 in regulating SOCS3 expression, affecting JAK-STAT pathway activity, and impacting cell proliferation and apoptosis.

Materials and methods

Main reagents and instruments

Human normal bile duct epithelial cells HIBEC were purchased from Sciencell, human biliary epithelial cell carcinoma cell lines QBC939 and HuCCT1 were purchased from Beijing Beina biological. HEK293T cells were purchased from the Chinese Academy of Sciences cell bank. DMEM medium and fetal bovine serum (FBS) were purchased from Gibco. Lipo 2000 was purchased from Invitrogen. PrimeScript™ RT reagent Kit was purchased from Takara. SYBR Green was purchased from Toyobo. MiR-221 inhibitor, miR-NC, and miR-221 mimic were purchased from Ribobio. Rabbit anti-human p-JAK2, p-STAT3, and β-actin antibodies were purchased from CST. Rabbit anti-human SOCS3 polyclonal antibody and HRP-conjugated secondary antibodies were purchased from Abacm. PMIR vector and double luciferase activity assay kit were purchased from Promega. Annexin V-FITC/PI double-stained cell apoptosis detection reagents were purchased from Beyotime. EdU-Alexa Fluor 488 cell proliferation assay kit was purchased from Molecular Probes.

Clinical information

Forty patients with intrahepatic cholangiocarcinoma (ICC) who underwent treatment in our hospital from January 2018 to June 2018 were recruited, consisting of 25 males and 15 females with an average age of 51.7 ± 10.5 years. The tumor and adjacent tissues removed during the operation were collected. All the tissue specimens were confirmed by pathological examination. The patients were informed about the study and the study was reviewed by the hospital ethics committee.

Cell culture

HIBEC, QBC939, and HuCCT1 cell lines were maintained in DMEM medium containing 10% FBS and cultured in 37°C and 5% CO_2. The cells were passaged at 1:5. Cells in the logarithmic growth phase were selected for experiments.

Dual luciferase reporter gene assay

The PCR product of the SOCS3 3'-UTR full-length fragment or mutant fragment was double-digested and then ligated into the pLUC vector. After sequencing, the plasmid was designated as pMIR-SOCS3-wt and pMIR-SOCS3-MUT. The HEK293T cells were transfected with pMIR-SOCS3-WT (or pMIR-SOCS3-MUT) together with miR-221 mimic (or miR-NC) by Lipofectamine 2000. Following incubation for 48 h, luciferase activity was measured by Dual-Luciferase Reporter Assay System kit in accordance with the instructions.

Cell transfection

QBC939 and HUCCT1 cells were divided into miR-NC group and miR-221 inhibitor group and cultured for 72 h followed by analysis of the expression of gene, protein, cell apoptosis, and proliferation.

qRT-PCR

Total RNA was extracted using Trizol reagent and reverse transcribed to cDNA. Using PrimeScript™ RT reagent Kit. The PCR was performed 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 5 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

Western blot

Total protein was extracted using RIPA lysis buffer and quantified by BCA assay. Forty μg protein was separated on SDS-PAGE, transferred to PVDF membrane, blocked with 5% skim milk and incubated with primary antibody at 4°C overnight (SOCS3, p-JAK2, p-STAT3, and β-actin at 1:2,000, 1:1,000, 1:1,000, and 1:8,000, respectively). After PBST washing, HRP conjugated secondary antibody was added and incubat-

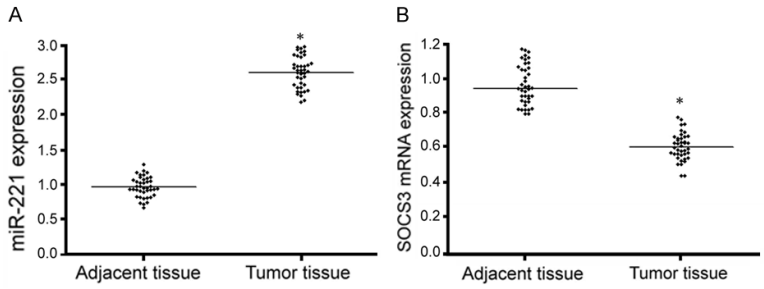

Figure 1. MiR-221 expression increased, while SOCS3 level declined in ICC tissue. A. qRT-PCR detection of miR-221 expression; B. qRT-PCR detection of SOCS3 mRNA expression. *P < 0.05, compared with adjacent tissue.

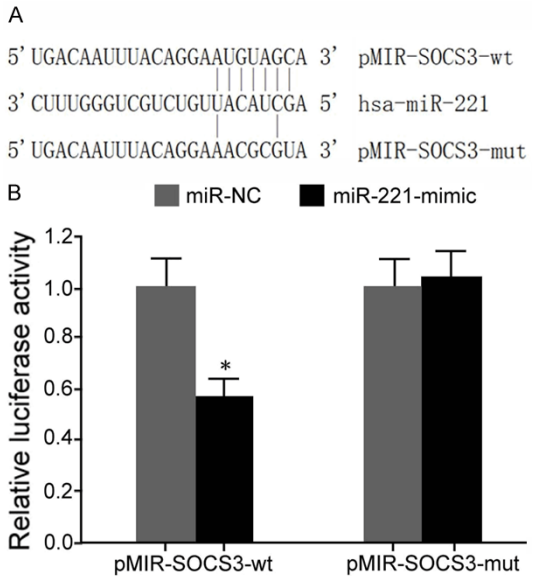

Figure 2. The targeted regulatory relationship between miR-221 and SOCS3. A. The complementary binding site between miR-221 and the 3'-UTR of SOCS3 mRNA. B. Dual luciferase reporter assay. *P < 0.05, compared with miR-NC.

ed for 60 min (1:8,000). Finally, the membrane was developed after addition of ECL chemiluminiscence reagent.

Cell apoptosis detection

Cells were collected and resuspended in 100 μl binding buffer followed by addition of 5 μl Annexin V-FITC and 5 μl PI and incubation at room temperature under dark for 15 min. After that, cell apoptosis was measured by flow cytometry.

EdU staining

EdU solution at 10 μM was added to cells in the logarithmic phase and incubated for 2 h followed by being cultured for 48 h and digested by trypsin. After being fixed with paraformaldehyde, the cells were incubated in 100 μL TritonX-100 at room temperature and kept in 500 μL reaction fluid for 30 min in the dark. Then, cell proliferation was assessed by FC 500 MCL flow cytometry.

Statistical analysis

SPSS 18.0 software was used for data analysis. Measurement data was displayed as mean ± standard deviation and the difference was compared by student t test or one-way ANOVA. P < 0.05 indicated a significant difference.

Results

MiR-221 expression increased, while SOCS3 level declined in ICC tissue

QRT-PCR showed that the expression of miR-221 was significantly increased in tumor tissues of intrahepatic cholangiocarcinoma (ICC) patients compared with adjacent tissues (**Figure 1A**). Moreover, qRT-PCR exhibited that SOCS3 mRNA level in tumor tissues of ICC patients was clearly lower than that in adjacent tissues (**Figure 1B**).

The regulatory relationship between miR-221 and SOCS3 mRNA

Bioinformatics analysis showed the complementary binding site between miR-221 and the 3'-UTR of SOCS3 mRNA (**Figure 2A**). Dual luciferase reporter gene assay showed that miR-221 mimic transfection markedly decreased the relative luciferase activity in HEK293T cells which were transfected by pMIR-SOCS3-WT but not by pMIR-SOCS3-MUT, confirming that SOCS3 was the target gene of miR-221 (**Figure 2B**).

MiR-221 and SOCS3 abnormal expressions in cholangiocarcinoma cells

QRT-PCR demonstrated that compared with HIBEC cells, the expression of miR-221 was apparently upregulated, while SOCS3 mRNA expression was significantly decreased in QBC939 and HuCCT1 cells (**Figure 3A**). Western blot analysis showed that the expression of

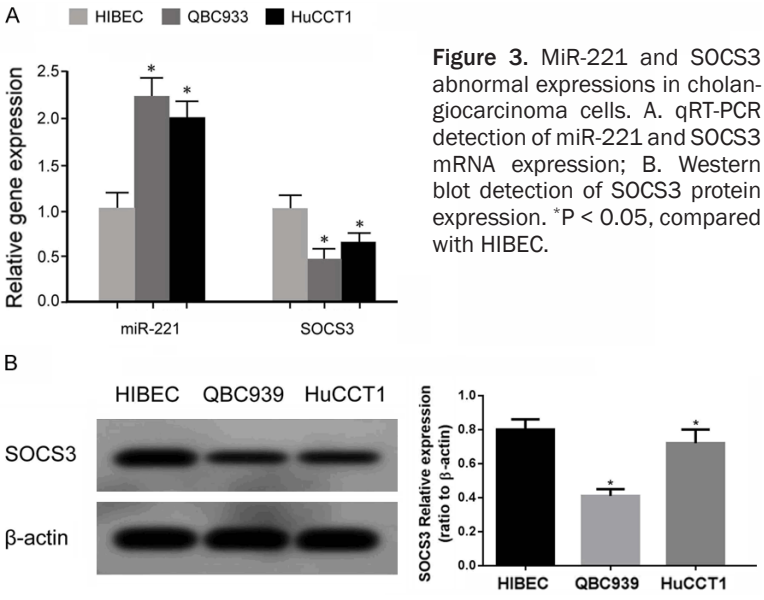

Figure 3. MiR-221 and SOCS3 abnormal expressions in cholangiocarcinoma cells. A. qRT-PCR detection of miR-221 and SOCS3 mRNA expression; B. Western blot detection of SOCS3 protein expression. *P < 0.05, compared with HIBEC.

SOCS3 protein in cholangiocarcinoma QBC939 and HuCCT1 cells was clearly lower than that of HIBEC cells (**Figure 3B**).

MiR-221 suppression promoted cholangiocarcinoma cell apoptosis and attenuated cell proliferation

QRT-PCR found that the expression of SOCS3 mRNA in QBC939 and HuCCT1 cells was clearly elevated in the miR-221 inhibitor transfection group compared with miR-NC transfection group (**Figure 4A**). Western blot analysis found that transfection of miR-221 inhibitor markedly enhanced SOCS3 protein expression, and apparently reduced p-JAK2 and p-STAT3 protein levels in QBC939 and HuCCT1 cells (**Figure 4B**). Flow cytometry revealed that the amount of cell apoptosis was significantly higher, while the cell proliferation ability was significantly weakened in the miR-221 inhibitor transfection group compared with the miR-NC transfection group (**Figure 4C** and **4D**).

Discussion

According to different anatomical locations, CCA can be divided into intrahepatic cholangiocarcinoma, hilar cholangiocarcinoma, and extrahepatic cholangiocarcinoma. In recent years, the incidence and death of cholangiocarcinoma are increasing year by year. The incidence rate is about 5/100,000 worldwide [19, 20]. Surgical resection is still the main treatment for CCA, whereas the 5-year survival rate is still lower than 30% [21, 22]. Therefore, exploring the abnormal changes of molecules in the pathogenesis of CCA is of great significance to help improving the early diagnosis, the therapeutic effect, and the prognosis.

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. Once activated, JAK will phosphorylate the tyrosine residue of receptor, leading to STAT complement STAT to the tyrosine phosphorylation site of the receptor complex via the SH2 domain. At this time, JAK kinase is spatially adjacent to STAT and phosphorylates its hydroxytyrosine. The phosphorylated activated STAT is separated from the receptor complex to form a dimer and gets transported from the cytoplasm to the nucleus, acting on specific DNA fragments and regulating gene transcription and expression [5]. SOCS negatively regulates the JAK-STAT signaling pathway and plays a critical role in maintaining cellular homeostasis. SOCS3 directly inhibits JAK kinase activity and STAT phosphorylation, thus negatively regulating JAK-STAT signaling [7]. SOCS3 is an important tumor suppressor gene, whose downregulation is associated with the occurrence, progression, and prognosis of various tumors, such as colorectal cancer [23], prostate cancer [9], and bladder cancer [10]. It was found that abnormal expression of SOCS3 is associated with the occurrence, progression, and drug resistance of cholangiocarcinoma [11, 12].

MicroRNA is an endogenous non-coding small RNA molecule in eukaryotes that can complementarily bind to the 3'-untranslated region (3'-UTR) of the target gene mRNA, leading to degradation of mRNA or inhibition of mRNA translation, thereby regulating several biological processes, such as cell survival, proliferation, apoptosis, migration. Abnormal expression and dysfunction of microRNA has received more and more attention in colorectal cancer [24], gastric cancer [25], and prostate cancer [26]. A number of studies observed that abnormal expres-

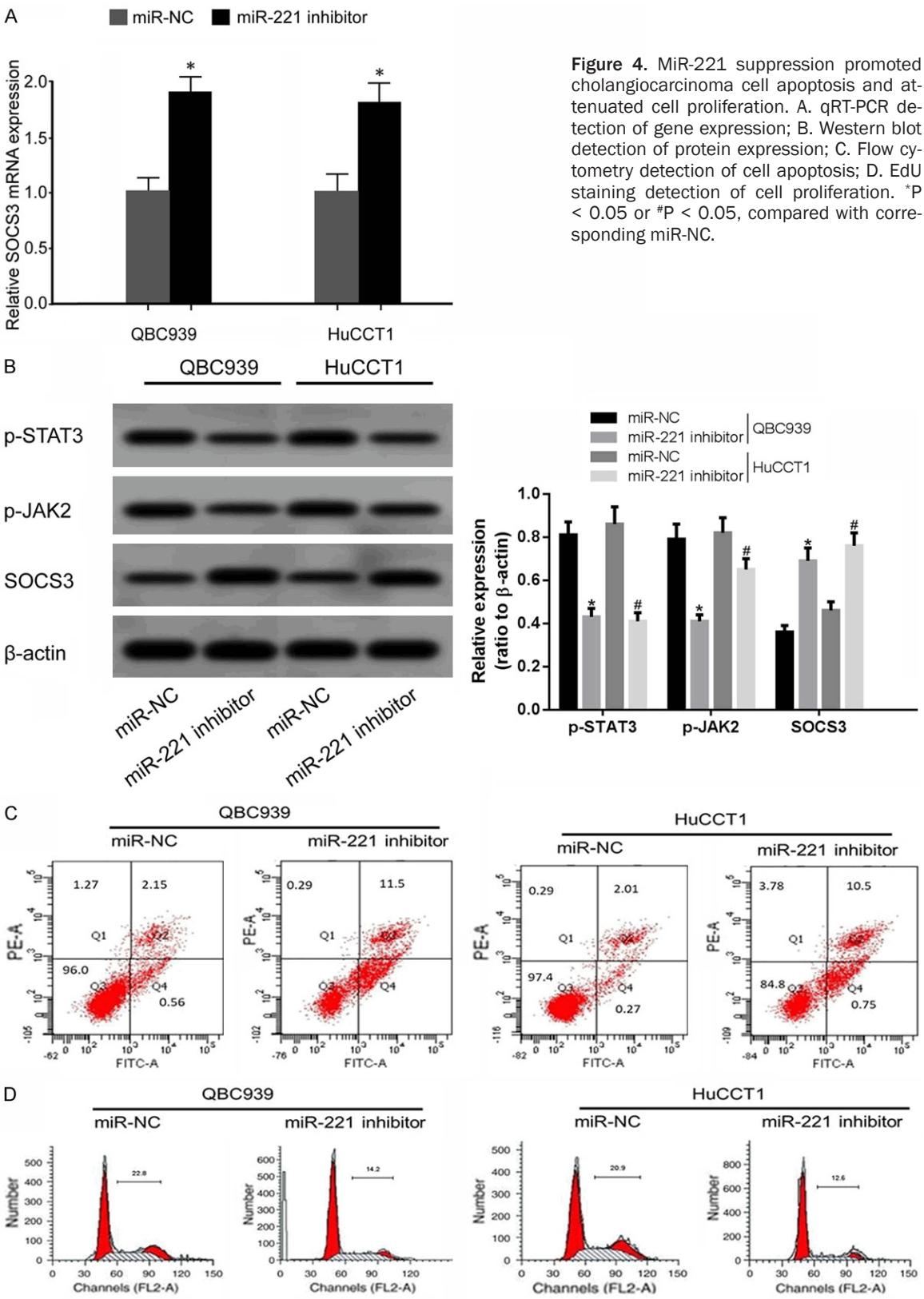

Figure 4. MiR-221 suppression promoted cholangiocarcinoma cell apoptosis and attenuated cell proliferation. A. qRT-PCR detection of gene expression; B. Western blot detection of protein expression; C. Flow cytometry detection of cell apoptosis; D. EdU staining detection of cell proliferation. *P < 0.05 or #P < 0.05, compared with corresponding miR-NC.

sion of miR-221 is closely associated with the occurrence and development of cholangiocarcinoma, suggesting that miR-221 may be a cancer-promoting factor [16-18]. Bioinformatics

analysis exhibited a targeted relationship between miR-221 and the 3'-UTR of SOCS3 mRNA. The present study assessed the role of miR-221 in regulating SOCS3 expression, affecting JAK-STAT pathway activity, and impacting cell proliferation and apoptosis.

In this study, we showed that transfection of miR-221 mimic significantly reduced the relative luciferase activity in pMIR-SOCS3-wt transfected HEK293T cells but not in pMIR-SOCS3-mut transfected HEK293T cells, indicating that there is a targeted regulatory relationship between miR-221 and SOCS3 mRNA. It was observed that compared with the adjacent tissues, miR-221 expression was clearly increased, while SOCS3 expression was markedly reduced in the tumor tissues of ICC patients, indicating that the increased expression of miR-221 plays a role in promoting the pathogenesis of cholangiocarcinoma through reducing SOCS3 expression. In addition, it was revealed that compared with normal bile duct epithelial cells, miR-221 level was also apparently upregulated, whereas SOCS3 level was significantly reduced in cholangiocarcinoma cells, which further suggested that miR-221 and SOCS3 promotes cancer and suppresses cancer, separately. In the study of the relationship between miR-221 and cholangiocarcinoma, Correa *et al.* showed that miR-221 expression in tumor tissues of ICC patients increased by 2.27 fold compared with adjacent tissues. In addition, compared with benign lesions, the expression of miR-221 was clearly elevated in plasma of ICC patients. Li *et al.* found that miR-221 level was significantly enhanced in extrahepatic cholangiocarcinoma (EHCC) compared with paracancerous bile duct tissue. Furthermore, the survival and prognosis of patients with higher miR-221 expression were significantly worse than those with lower miR-221 expression. This study exhibited abnormally elevated miR-221 expression in tumor tissues of ICC patients, suggesting that its cancer-promoting effect is consistent with Correa and Li [18]. In the study of the relationship between SOCS3 and cholangiocarcinoma, Wang *et al.* demonstrated that the expression of SOCS3 in tumor tissues of patients with cholangiocarcinoma was significantly lower than that of normal bile duct tissues. SOCS3 expression was lower in tumor cells with lower the degree of differentiation. The prognosis of patients with lower SOCS3 expression was worse than the patients with higher SOCS3 expression. Isomoto *et al.* [27] showed that the methylation level of the SOCS3 gene promoter region in cholangiocarcinoma cells significantly increased, resulting in significantly enhanced SOCS3. This leads to an clearly elevated IL-6/STAT3 pathway activity and weakened the apoptosis sensitivity. These findings confirmed the role of the SOCS3 gene in tumorigenesis in cholangiocarcinoma, which was similar to the results in this study.

To further assess miR-221's role in the regulation of the biological effects of cholangiocarcinoma cells, this study transfected miR-221 inhibitor to cholangiocarcinoma cells and observed its impact on the biological effects. The results demonstrated that transfection of miR-221 inhibitor obviously upregulated SOCS3 gene expression, promoted the apoptosis of cholangiocarcinoma QBC939 and HuCCT1 cells, and weakened the cell proliferation ability. Li *et al.* found that miR-221 expression in cholangiocarcinoma QBC939, HuCCT1, RBE, and HCCC9810 cells was markedly higher than that of normal bile duct epithelial cells. There is a targeted regulation relationship between miR-221 and PTEN as a tumor-promoting gene. MiR-221 inhibitor transfection reduced miR-221 expression, which can significantly elevate the expression of tumor suppressor gene PTEN and inhibit epithelial-mesenchymal transition that attenuates the invasive ability of cholangiocarcinoma cells. Zhou *et al.* [28] reported that overexpressed SOCS3 can significantly inhibit the activation of IL-6/STAT3 pathway, attenuate the EMT process induced by IL-6, reduced N-cadherin and vimentin expressions, enhance E-cadherin level, and decreas the migration and invasion of cholangiocarcinoma RBE and HCCC9810 cells. At present, there is no research on the impact of miR-221 on cholangiocarcinoma cells by regulating SOCS3. The present study evaluated the targeted regulation between miR-221 and SOCS3, revealing that miR-221 regulates SOCS3 expression, affects JAK-STAT pathway activity, and influences the proliferation and apoptosis of cholangiocarcinoma cells. However, whether miR-221 has the ability of regulating the biological process of cholangiocarcinoma *in vivo* is still unclear, and further animal experiments are needed.

Conclusion

Increased expression of miR-221 and decreased expression of SOCS3 are associated

with the pathogenesis of cholangiocarcinoma. MiR-221 can regulate the proliferation and apoptosis of cholangiocarcinoma cells by targeted inhibition of SOCS3 expression and affecting JAK-STAT pathway activity.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Kai Cheng, Department of Gastrointestinal Surgery, Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Edong Healthcare Group, No.141, Tianjin Road, Huangshi 435000, Hubei, China. Tel: +86-0714-6233931; Fax: +86-0714-6233931; E-mail: xianhuidou82@163.com

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