MiR-155 regulates lymphoma cell proliferation and apoptosis through targeting SOCS3/JAK-STAT3 signaling pathway

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Abstract. – OBJECTIVE: Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway participates in regulating cell proliferation, differentiation, and apoptosis, and related to lymphoma. Suppressors of cytokine signaling 3 (SOCS3) is a negative regulator of the JAK-STAT signaling pathway. SOCS3 reduction and miR-155 up-regulation are associated with lymphoma pathogenesis. Bioinformatics analysis showed the complementary binding site between miR-155 and SOCS3. This study aimed to investigate the role of miR-155 in regulating SOCS3/JAK-STAT signaling pathway and affecting diffuse large B cell lymphoma (DLBCL) cell proliferation and apoptosis.

PATIENTS AND METHODS: DLBCL tumor sample was collected from the patients in our hospital. Lymphatic tissue derived from reactive lymphoid hyperplasia patients were selected as control. MicroRNA-155 (MiR-155) and SOCS3 expressions were detected. Dual luciferase assay was used to verify the targeted relationship between miR-155 and SOCS3. OCI-LY10 cells were cultured in vitro and divided into five groups, including miR-NC, miR-155 inhibitor, pIRES2-Blank, pIRES2-SOCS3, and miR-155 + pIRES2-SOCS3 groups. SOCS3, p-JAK1, p-JAK2, p-STAT3, and Survivin expressions were tested. Cell apoptosis and proliferation were detected by flow cytometry.

RESULTS: MiR-155 expression significantly increased, while SOCS3 level declined in DLBCL tissue compared with control. MiR-155 targeted regulated SOCS3 expression. MiR-155 inhibitor and/or pIRES2-SOCS3 transfection markedly up-regulated SOCS3 expression, reduced p-JAK1, p-JAK2, p-STAT3, and Survivin levels, attenuated cell proliferation, and enhanced cell apoptosis in OCI-LY10 cells.

CONCLUSIONS: Down-regulation of miR-155 inhibited DLBCL cell proliferation and facilitated apoptosis through up-regulating SOCS3 expression to suppress JAK-STAT3 signaling pathway.

Key Words: MiR-155, SOCS3, JAK-STAT3, Lymphoma, Proliferation, Apoptosis.

Introduction

Lymphoma is a kind of malignant tumor that takes place in the lymphoid and hematological system, such as lymph nodes, spleen, thymus, and extranodal lymphatic tissues and organs. Lymphoma is associated with immune cell canceration, thus belonging immune system malignant tumor. Lymphoma contains Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL). NHL is the major type accounting for more than 90% of lymphoma. Although great development has been achieved in surgical resection, whole body standard chemotherapy, local radiotherapy, and biological immune therapy, about 60% patients still obtain non-ideal curative effect.

Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway excessive activation is closely related to multiple cancers occurrence, development, invasion, and metastasis by promoting cell proliferation and antagonizing apoptosis. Suppressors of cytokine signaling 3 (SOCS3) is one of the members of SOCS family that can block cytokines response to negatively regulate JAK-STAT signaling pathway. SOCS3 reduction and miR-155 up-regulation are associated with lymphoma pathogenesis. Bioinformatics analysis showed the complementary binding site between miR-155 and SOCS3. This study aimed to investigate the role of miR-155 in regulating SOCS3/JAK-STAT signaling pathway and affecting diffuse large B cell lymphoma (DLBCL) cell proliferation and apoptosis.

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an oncogene role in lymphoma\textsuperscript{18} and related to lymphoma disease degree and poor prognosis\textsuperscript{19}. Bioinformatics analysis showed the complementary binding site between miR-155 and SOCS3. This study aimed to investigate the role of miR-155 in regulating SOCS3/JAK-STAT signaling pathway and affecting lymphoma cell proliferation and apoptosis.

Patients and Methods

Main Reagents and Materials

Human diffuse large B cell lymphoma (DLBCL) OCI-LY10 cells were purchased from Shanghai Yubo biological technology co., Ltd. (Shanghai, China). RPMI-1640 medium, Opti-MEM, penicillin, and streptomycin were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). FBS was got from Gemini Bio Products (Woodland, CA, USA). EasyPure RNA Kit and Real-time PCR reagent TransScript Green One-Step qRT-PCR SuperMix were obtained from TransGen Biotech (Beijing, China). Mouse anti-human Survivin, JAK1, p-JAK1, JAK2, and p-JAK2 antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-human STAT3, phosphorylated STAT3 (p-STAT3), SOCS3, and β-actin antibodies were bought from Cell Signaling Technology Inc. (Beverly, MA, USA). Goat anti-rabbit and rabbit anti-mouse secondary antibodies were got from Bio-Rad Laboratories (Hercules, CA, USA). Annexin-V/PI apoptosis detection kit was purchased from Affinity BioReagents (Hangzhou, China). Cell proliferation detection kit Click-IT\textsuperscript{8} EdU Alexa Fluor 488 Flow Cytometry Assay Kit was obtained from Molecular Probes (Eugene, OR, USA). Dual-Glo\textsuperscript{4} Luciferase Assay System and pMIR luciferase reporter gene plasmid were provided by Promega (Madison, WI, USA). Lipofectamine 2000 was derived from Invitrogen Life Technologies (Carlsbad, CA, USA).

Clinical Information

A total of 29 DLBCL patients received treatment between May 2016 and Oct 2016 in Yan’an People’s Hospital of Shaanxi Province and another 32 patients suffered from reactive lymphoid hyperplasia were enrolled. The lymphatic tissue and tumor tissue were collected. The study was approved by the Ethics Committee of Yan’an People’s Hospital of Shaanxi Province. All the patients signed the informed consent.

Cell Culture

OCI-LY10 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were passaged at 1:4.

Dual-luciferase Reporter Gene Assay

The PCR products containing the full length of SOCS3 gene 3’-UTR segment were cloned to pMIR. Next, it was transformed into DH5\textalpha competent cells and sequenced to select the plasmid with correct sequence. Then, pMIR-SOCS3-3’-UTR-wt (or pMIR-SOCS3-3’-UTR-mut) was co-transfected with HEK293T cells using Lipofectamine 2000 together with miR-155 mimic (or miR-NC, or miR-155 inhibitor). The luciferase activity was detected according to the Dual-Glo\textsuperscript{4} Luciferase Assay manual after cultured for 48 h.

SOCS3 Overexpression Plasmid Construction

The CDS region fragment of SOCS3 gene was amplified and connected to pIRES2 plasmid after digested by Xho I and BamH I. Then it was transformed into JM109 competent cells and screened by penbritin. Next, the plasmid was extracted and sequenced. It was named as pIRES2-SOCS3, while pIRES2-Blank was treated as control.

Cell Transfection and Grouping

OCI-LY10 cells were cultured in vitro and divided into five groups, including miR-NC, miR-155 inhibitor, pIRES2-Blank, pIRES2-SOCS3, and miR-155 + pIRES2-SOCS3 groups. Nucleotide fragments and Lipofectamine 2000 were added to Opti-MEM and incubated at room temperature for 30 min, respectively. Then they were added to the cells cultured in Opti-MEM. After 6 h incubation, the medium was changed back to RPMI 1640 medium containing 10% FBS and 1% penicillin-streptomycin. The cells were used for detection after 72 h.

qRT-PCR

Total RNA was extracted using EasyPure RNA Kit and adopted for PCR reaction by TransScript Green One-Step qRT-PCR SuperMix. The reaction system contained 1 μg RNA template, 0.3 μM primers, 10 μl 2×TransStart Tip Green qPCR SuperMix, 0.4 μl RT Enzyme Mix, 0.4 μl Dye II, and ddH\textsubscript{2}O. The reverse transcription condition was 37°C for 15 min and 98°C for 5 min. The PCR reaction was composed of 45°C reverse transcription for 5 min, 94°C pre-denaturation for 30 s, followed...
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by 40 cycles of 94°C for 5 s and 60°C for 30 s. Re-
al-time PCR was performed on Bio-Rad CFX96/
CFX connect to test the relative expression.

Western Blot
Total protein was extracted by RIPA from
cells. A total of 40 μg protein was separated by
8%-10% sodium lauryl sulfate-polyacrylamide
gel electrophoresis (SDS-PAGE) for 3 h and trans-
ferred to membrane. Next, the membrane was
blocked and incubated with primary antibody at
4°C overnight (SOCS3, JAK1, p-JAK1, JAK2,
p-JAK2, STAT3, p-STAT3, Survivin, and β-actin
1:800, 1:5000, and 1:10000, respectively). Then,
the membrane was incubated with horseradish
peroxidase (HRP) labeled secondary antibody
(1:10000) for 60 min after washed by phosphate
buffered saline-tween 20 (PBS-T) for three times.
At last, the protein expression was detected by
enhanced chemiluminescence (ECL).

EdU Staining
The cells were added to 10 μM EdU solution at
37°C for 120 min. After incubated for 48 h, the cells
were digested by trypsin and collected. After wa-
sed with phosphate-buffered saline (PBS) contai-
ning 1% bovine serum albumin (BSA) and fixed in
100 µl Click-iT® fixative for 15 min, the cells were
penetrated by 100 μl 1×Click-iT® saponin-based
permeabilization and wash reagent at room tempe-
ration for 15 min. Next, the cells were incubated
in 500 μl reaction liquid containing PBS, CuSO₄,
Alexa Fluor 488, and Buffer additive at room tem-
perature for 30 min. Then the cells were washed by
3 ml 1×Click-iT® saponin-based permeabilization
and wash reagent for 1 time. At last, the cells were
resuspended in 500 μl 1×Click-iT® saponin-based
permeabilization and wash reagent and tested by
Beckmann FC 500 MCL/MPL flow cytometry.

Flow Cytometry
The cells were resuspended in 500 μl binding
buffer and incubated in 5 μl Annexin V-FITC and

Figure 1. MiR-155 up-regulated, while SOCS3 reduced in DLBCL tissue. (A) qRT-PCR detection of miR-155 and SOCS3
mRNA expressions. (B) Western blot detection of SOCS3 protein expression.

Figure 2. MiR-155 targeted regulated SOCS3 expression. (A) The binding site between miR-365 the 3’-UTR of DJ-1
mRNA. (B) Dual luciferase assay. *p<0.05, compared with miR-NC.
5 μl PI avoid of light for 15 min. Next, the cells were added with 5 μl PI and tested on Beckmann FC 500 MCL/MPL flow cytometry to evaluate cell apoptosis.

**Statistical Analysis**

All data analyses were performed by the SPSS 18.0 software (Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by t-test. \( p < 0.05 \) was considered as statistical significance.

**Results**

**MiR-155 Up-Regulated, While SOCS3 Reduced in DLBCL Tissue**

Quantitative RT-PCR (qRT-PCR) showed that SOCS3 mRNA significantly reduced, while miR-155 elevated in the tumor tissue from DLBCL patients compared with control (Figure 1A). Western blot revealed that SOCS3 protein markedly reduced in DLBCL tissue compared with control, indicating miR-155 up-regulation may play a role in reducing SOCS3 and promoting DLBCL pathogenesis (Figure 1B).

**MiR-155 Targeted Regulated SOCS3 Expression**

MicroRNA.org online prediction showed the targeted binding site between miR-155 and 3’-UTR of SOCS3 mRNA (Figure 2A). Dual luciferase assay revealed that miR-155 mimics or inhibitor transfection significantly declined or increased the relative luciferase activity of HEK293 cells, indicating the regulatory relationship between miR-155 and SOCS3 mRNA.

**Discussion**

JAK-STAT signaling pathway is first found in interferon study. It is discovered to be activated by multiple cytokines and growth factors, and mediates various cytokines signal transduction. JAK-STAT signaling pathway is mainly composed of tyrosine kinase associated receptor, STAT, and JAK6.

JAK-STAT signaling pathway activation is associated with tumor pathogenesis by up-regulating Bel-2, Survivin, Cyclin D1, and c-Myc. As an oncogene transcription factor, STAT3 is an important member of STAT family that plays a critical role in a variety of cancer pathogenesis. JAK-STAT3 participates in multiple biological processes, such as cell proliferation, cycle, and apoptosis, thus, is associated with tumor occurrence, development, invasion, and metastasis, including lung cancer, colorectal cancer, prostate cancer, and pancreatic cancer. As the strongest member of SOCS family, SOCS3 plays a negative regulatory role in JAK-STAT3 signaling pathway by inhibiting JAK kinase binding with receptor to suppress JAK phosphorylation or...
competitive binding with JAK to restrain STAT3 phosphorylation via N-terminal kinase inhibitory region (KIR)11,13. SOCS3 is related to multiple cancers' occurrence, metastasis, drug resistance, and poor prognosis, such as breast cancer11, prostate cancer12, lung cancer13, and pancreatic cancer14. SOCS3 expression significantly reduced in NHL tissue, indicating that SOCS3 plays a tumor suppressor gene role in NHL14. MiR-155 was found to play an oncogene role in lymphoma15 and related to lymphoma disease degree and poor prognosis16. Bioinformatics analysis showed the complementary binding site between miR-155 and SOCS3. This research aimed to investigate the role of miR-155 in regulating SOCS3/JAK-STAT signaling pathway and affecting lymphoma cell proliferation and apoptosis.

Our investigation showed that miR-155 mimics or inhibitor transfection significantly declined or increased the relative luciferase activity of HEK293 cells, indicating the regulatory relationship between miR-155 and SOCS3 mRNA. SOCS3 significantly reduced, while miR-155 elevated in the tumor tissue from DLBCL patients compared with control, indicating miR-155 up-regulation may play a role in reducing SOCS3 and promoting DLBCL pathogenesis. Slezak-Prochazka et al25 reported that miR-155 expression in B-cell lymphoma abnormally up-regulated. Zhong et al26 demonstrated that miR-155 increased in DLBCL compared with reactive lymphatic hyperplasia and was correlated with complete remission, overall response rate, and poor prognosis. Munch-Petersen et al27 found miR-155 overexpressed limited in tumor tissue of DLBCL by nucleic acid hybridization in situ technique. Huang et al28 revealed that miR-155 level markedly elevated in activated B-cell-like (ABC) DLBCL tissue with lower invasion and malignancy. Moreover, miR-155 expression was apparently higher in OCI-Ly3 and OCI-Ly10 compared with that in SU-DHL6 and SU-DHL16. Merkel et al28 demonstrated that inhibition of miR-155 suppressed lymphoma cell proliferation in vitro and in vivo. In this work, miR-155 downregulation markedly attenuated OCI-Ly10 cell malignancy. Molavi et al24 reported that SOCS3 over-expression apparently suppressed STAT3 phosphorylation, downregulated cyclin D1, BCL2, and BCL-XL expressions, and induced apoptosis in B-cell lymphoma cell line Mino and Rec-1. At present, the role of miR-155 in regulating SOCS3 expression and DLBCL cell proliferation and apoptosis has not been reported. This investigation showed that miR-155 plays a role in suppressing SOCS3 expression and promoting DLBCL pathogenesis. Down-regulation of miR-155 increased SOCS3 expression and blocked JAK-STAT3 signal transduction to inhibit lymphoma cell proliferation and accelerated cell apoptosis. This study only collected DLBCL tissue and investigated the impact of miR-155 on SOCS3-JAK-STAT3 signaling pathway and DLBCL cell proliferation and apoptosis, while whether miR-155 may regulate other types of B-cell lymphoma is still unclear.

Conclusions

MiR-155 over-expression plays a role in inhibiting SOCS3 and promoting DLBCL pathogenesis. Down-regulation of miR-155 inhibited DLBCL cell proliferation and facilitated apoptosis through up-regulating SOCS3 expression to suppress JAK-STAT3 signaling pathway.
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Conflict of interest
The authors declare no conflicts of interest.

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