

MiR-93 blocks STAT3 to alleviate hepatic injury after ischemia-reperfusion

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Abstract. – OBJECTIVE: Signal transducer and activator of transcription 3 (STAT3) is correlated with ischemia-reperfusion (I-R) injury. The previous studies showed a decreased miR-93 expression after I-R injury of heart or brain organs, but without knowledge in liver tissues. This study aims to investigate effects of MiR-93 on the hepatic injury after ischemia/reperfusion.

MATERIALS AND METHODS: Rat liver I-R model was generated. Liver function indexes including alanine transaminase (ALT) and aspartate aminotransferase (AST) were quantified, and serum tumor necrosis factor α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) levels were quantified. Hepatic tissue apoptosis was measured by transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL), and expression of microRNA-93 (miR-93), STAT3, and phosphorylated STAT3 (p-STAT3) were measured. Dual luciferase reporter gene assay confirmed targeted relationship between miR-93 and STAT3. Agomir or miR-93 agomir was injected into the peritoneal cavity of I-R model, followed by ALT and AST assays. Serum levels of TNF- α , IL-1 β , and IL-6 were measured, followed by TUNEL assay for comparing STAT3 and p-STAT3 expression.

RESULTS: Comparing to sham group, I-R group rat showed significantly elevated serum ALT, AST, TNF- α , IL-1 β , and IL-6 contents, along with significantly elevated hepatic cell apoptosis, plus decreased miR-93 expression, whilst STAT3 and p-STAT3 expression was enhanced. Intraperitoneal injection of miR-93 agomir significantly decreased STAT3 or p-STAT3 expression, and decreased cell apoptotic rate. Serum levels of ALT, AST, TNF- α , IL-1 β , and IL-6 were significantly decreased, accompanied by improved liver function.

CONCLUSIONS: Hepatic I-R injury is accompanied by miR-93 down-regulation, plus STAT3 up-regulation. Overexpression of miR-93 significantly depressed STAT3 expression in liver I-R injury, alleviated hepatic injury or apoptosis, decreased inflammatory response, and improved liver function.

Key Words

MiR-93, STAT-3, Liver tissues, Ischemia-reperfusion injury, Inflammatory response, Cell apoptosis.

Introduction

Ischemia-reperfusion (I-R) injury is one common pathological process within various liver diseases and is frequently caused by ischemia shock, liver transplantation, hepatectomy, or intervention therapy¹⁻³. Hepatic I-R injury is a common critical condition in clinics, and still lacks effective treatment approach. Therefore, the development of pathogenesis mechanism of hepatic I-R, and investigation of signal modulating molecules with abnormal change during injury course, are of critical importance for improving treatment efficiency and patient prognosis.

Janus kinase (JAK)-signal transducer and activator of transcription (STAT) is one widely studied signal transduction pathway, and is widely distributed in various tissues and organs. STAT protein family consists of 7 members, among which STAT3 is the most widely studied one and is the most important member. STAT3 expression or function abnormality plays crucial roles in inducing I-R injury of various tissue/organs including heart⁴, brain⁵, and kidney⁶. A previous study⁷ showed that during hepatic I-R injury, STAT3 expression and function are strongly potentiated, suggesting close correlation between STAT3 and hepatic I-R injury. MicroRNA (miR) is one group of endogenous non-coding small molecule single stranded RNA with 22-25 nt length, and can regulate target gene expression via degrading or inhibiting translation of mRNA by the means of complementary base

paring. Expression and function abnormality of miR molecules have drawn lots of research interests in I-R injury of multiple organs including heart⁸, spinal cord⁹, and kidney¹⁰. Scholars^{11,12} showed decreased miR-93 expression in heart or brain tissues after I-R injury. More importantly, miR-93 exerts protective roles in I-R injury, but leaving its regulatory role in hepatic I-R injury unclear. Bioinformatics analysis showed the existence of targeted complementary binding sites between miR-93 and 3'-UTR of STAT3 mRNA, indicating a possible regulatory correlation between those two factors. We thus established a rat hepatic I-R model, on which expressional change of miR-93 and STAT3 was examined. We further interfered miR-93 expression in living rats, and investigated the role of miR-93 in mediating STAT3 expression and hepatic I-R injury.

Materials and Methods

Major Reagent and Materials

Healthy male adult Sprague-Dawley (SD) rats (6 weeks age, body weight 220-240 g) were purchased from Silaike Laboratory Animal Inc (Shanghai, China). PrimeScriptTM RT reagent Kit and SYBR Green were purchased from Takara (Dalian, China). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) test kits were purchased from Nanjing Jiancheng Bioengineer Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) were purchased from RayBiotech Inc. (Norcross, GA, USA). MiRNA-93 agomir, miRNA agomir control, miR-NC and miR-93 mimic were purchased from RioBio (Shanghai, China). transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) apoptosis kit, caspase-3 activity assay kit, and horseradish peroxidase (HRP) conjugated secondary antibody were purchased from Beyotime Biotechnology (Shanghai, China). Rabbit anti-rat STAT3, p-STAT3, and beta-actin monoclonal antibody were purchased from Abcam Biotechnology (Cambridge, MA, USA). Luciferase activity assay kit Dual-Glo Luciferase Assay System and pMIR-REPORT Luciferase plasmid were purchased from Youbao Bio (Shanghai, China). Transfection kit Lipo2000 was purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA).

Liver I-R Injury Model Construction

SD rats were divided into two groups: I-R group and Sham group (n=5 each). One week before experiment, rats were acclimated for feeding and were fasted for 12 h before surgery with water *ad libitum*. Rats were anesthetized by intraperitoneal injection of 10% hydrate chloral and were placed in supine position. Local skin was sterilized, and abdominal incision was made below xiphoid process. The hepatic portal was exposed and hepatic pedicle was freed. Portal vein and hepatic artery branch between left and middle hepatic lobules were clapped. The darkening of liver tissue color on those regions with blocked blood supply indicated hepatic ischemia. 45 min later, artery clamp was relieved to restore blood supply and reperfusion. The abdominal cavity was then closed. In Sham group, hepatic portal was exposed without clapping the vein. At 6 h, 12 h, and 24 h after surgery, serum AST, ALT, TNF- α , IL-1 β , and IL-6 contents were measured. At 6 h, 12 h, and 24 h after surgery, liver tissues were harvested, and Western blot was performed to measure protein expression. 24 h after surgery, TUNEL kit was employed to measure apoptosis of hepatic cells.

This study was approved by the Ethics Committee of the Hubei Xianning Ma Tang Rheumatism Hospital, Xianning, China.

Grouping of Experimental Animals

Rat I-R hepatic injury model was generated and was randomly divided into two groups: miRNA agomir control and miRNA-93 agomir group (n=5 per group). Both groups received 20 nmol agomir control or miRNA-93 agomir *via* intraperitoneal injection before surgery. 24 h after surgery, serum was collected to measure AST, ALT, TNF- α , IL-1 β , and IL-6 contents. 24 h after surgery, liver tissues were harvested to measure protein expression by Western blot. The caspase-3 activity was measured by spectrometry.

Liver Function Index AST and ALT Assay

At 0 h, 12 h, and 24 h after surgery, 2 ml venous blood samples were harvested from inferior vena cava, and were kept in 4°C overnight. Blood samples were centrifuged at 400 $\times$ g for 10 min, and the upper supernatant was saved and kept at -80°C fridge. An automatic biochemical analyzer (Mode: AU800, Olympus, Japan) was employed to measure the level of serum AST and ALT.

ELISA for Measuring TNF- α , IL-1 β , and IL-6 Contents

ELISA was performed following the manual instruction. In brief, 96-well ELISA plate with TNF- α , IL-1 β , and IL-6 antibody pre-coating was added with 100 μ l gradient diluted TNF- α , IL-1 β , and IL-6 standard samples or serum samples. After 2.5 h room temperature incubation, 100 μ l 1 $\times$ wash solution was added into each well for 4 times of washing. 100 μ l biotin labelled secondary antibody was then added for 60 min at room temperature. After removing secondary antibody, 100 μ l 1 $\times$ wash solution was added into each well for 4 times of washing, followed by 100 μ l 3,3',5,5'-tetramethylbenzidine (TMB) One-Step Substrate Reagent at 30 min room temperature incubation. 50 μ l Stop Solution was added into each well and absorbance value at 450 nm was measured.

TUNEL for Hepatic Tissues Apoptosis

Twenty-four hours after surgery, rat liver tissues were harvested to prepare frozen sections. Following the manual instruction of TUNEL assay kit, tissues were fixed in 4% paraformaldehyde for 30 min, and were washed in phosphate-buffered solution (PBS) twice. Tissues were permeabilized at room temperature for 5 min, and were incubated in TUNEL reaction buffer containing 10% TdT enzyme plus 90% fluorescent labelling solution. Tissues were then washed in PBS for three times, and were mounted in anti-bleaching solution. Fluorescent microscope was used for observation.

Caspase-3 Activity Assay

Following the manual instruction of caspase-3 activity assay kit, pNA standard samples were prepared and A405 values were measured to plot standard curves. Liver tissues were lysed on ice, and the supernatant was transferred into new pre-cold tubes for further use. Test buffer, samples and Ac-DEVD-pNA were sequentially added into 96-well plate, which was incubated at 37°C for 2 h. When founding significant color change, microplate reader was used to measure A405 in test samples, which can reflect caspase-3 activity.

Dual Luciferase Gene Reporter Assay and Recombinant Plasmid Construction

Using RNA of HEK293T cells as the template, 3'-UTR of STAT3 mRNA containing targeted binding sites or its mutant form was amplified, and was digested in Sac I and Hind III enzymes at 37°C for 4 h. pMIR plasmid was also digested using the same enzyme pair. Products were purified

in 1.5% agarose gel electrophoresis, and were ligated with digested plasmids at 16°C overnight. Ligated products were transformed into DH5 α competent cells, which were inoculated into penicillin-containing plate for 37°C overnight incubation. Single positive clone was picked and cultured at medium overnight at 37°C. Plasmid was extracted and sequenced to determine targeted sequence, and was named as pMIR-STAT3-wt or pMIR-STAT3-mut.

Luciferase Reporter Gene Assay

1 $\times$ 10⁵ HEK-293T cells were seeded into 24-well plate for 24 h culture. Lipo 2000 was used to co-transfect 100 ng pMIR-STAT3-wt (or pMIR-STAT3-mut), 900 ng miR-93 mimic (or miR-NC) and 50 ng pRL-null renilla luciferase into HEK293T cells for 48 h continuous incubation. Dual-Glo Luciferase Assay System was used to measure dual luciferase activity. In brief, 100 μ l passive lysis buffer was added into each well of 24-well plate for mixture. Fluorescent meter was used to measure firefly luciferase activity. 100 μ l renilla luciferase reagent was then added for measuring fluorescent values. Relative activity was calculated by firefly luciferase activity divides renilla luciferase activity.

Quantitative Real-Time PCR (qRT-PCR) for Gene Expression

Liver tissues were lysed by TRIzol buffer. PrimeScriptTM RT reagent kit was used to generate complementary DNA (cDNA) from RNA template by reverse transcription. Using cDNA as the template, PCR was performed using TaqDNA polymerase. In a 10 μ l reaction system, one added 5.0 μ l 2 $\times$ SYBR Green Mixture, 0.5 μ l of forward and reverse primers (5 μ M/l), 1 μ l cDNA, and ddH₂O filling up to 10.0 μ l. Reverse transcription was performed under the conditions: 50°C for 15 min, and 85°C 5 min. PCR parameters were: 95°C denature for 5 min, followed by 40 cycles each consisting of 95°C 15 s and 60°C 1 min. PCR was performed on Bio-Rad CFX96 cycler (Bio-Rad Laboratories, Hercules, CA, USA). Primer sequences were: miR-93 Forward primer: 5'-AGTCT CTGGC TGACT TACAT CACAC-3'; Reverse primer: 5'-CTACT CACAA AACAG GAGTG GAATC-3'; STAT3 forward primer: 5'-CCTGA AGCTG ACCCA GGTA-3'; STAT3 reverse primer: 5'-CCTGA AGCTG ACCCA GGTA-3'; Beta-actin forward primer: 5'-TGAGC GAGGC TACAG CTT-3'; Beta-actin reverse primer: 5'-TCCTT GATGT CGC-ACGAT TT-3'.

Table I. ALT and AST results at all time points after surgery.

Index	Group	6 h post-option	12 h post-option	24 h post-option
ALT (U/L)	Sham group	62.7±11.3	59.5±13.2	66.2±12.8
	I-R group	161.6±16.3*	274.3±22.8*	419.5±38.8*
AST (U/L)	Sham group	114.5±13.6	121.7±15.8	109.8±15.3
	I-R group	269.2±31.7*	388.9±44.5*	516.4±58.6*

* $p < 0.05$ comparing to Sham group.

Western Blot for Protein Expression Assay

Each of 50 ng liver tissues were mixed with 1 ml RIPA lysis buffer for 30 min iced incubation. Lysate was centrifuged at 10000 ×g for 10 min, and protein supernatant was transferred into new tubes. 40 µg samples were loaded and were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred into polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in phosphate-buffered solution Tween-20 (PBST) containing 5% defatted milk powder for 60 min at room temperature. Primary antibody (STAT3 at 1:2000, p-STAT3 at 1:800 and beta-actin at 1:10000) was added for 4°C overnight incubation. Excess antibody was removed and secondary antibody (1:15000 dilution) was added for 60 min room temperature incubation. Enhanced chemiluminescent (ECL) approach was used to develop the membrane, followed by exposure and fixation. The film was scanned to save data.

Statistical Analysis

SPSS 18.0 software was used for data analysis (SPSS Inc., Chicago, IL, USA). Measurement data were presented as mean ± standard deviation (SD). The Student's *t*-test was utilized for the statistical analysis between two groups. Tukey's post-hoc test was used to validate the ANOVA for

comparing measurement data among groups. A statistical significance was defined when $p < 0.05$.

Results

I-R Rats Showed Liver Dysfunction and Elevated Inflammatory Factors Pus Apoptosis

Liver function assay showed that, comparing to Sham group rats, I-R group rats showed significantly elevated serum ALT and AST contents. With elongated I-R time, serum ALT and AST levels were gradually increased, whilst Sham group rats showed relatively lower serum ALT and AST at all time points after surgery (Table I). ELISA results showed that, comparing to Sham group, I-R rats had remarkably elevated serum TNF- α , IL-1 β , and IL-6 contents. With elongated I-R time, serum TNF- α , IL-1 β , and IL-6 levels were gradually elevated, whilst Sham group showed relatively lower serum TNF- α , IL-1 β , and IL-6 levels at all time points after surgery (Table II). Spectrometry showed higher caspase-3 enzymatic activity at all time points of I-R rat liver tissues comparing to Sham group. With elongated time, caspase-3 activity was further potentiated, and reached the peak level at 24 h after surgery (Figure 1A). TUNEL at 24 h after surgery showed significantly higher apoptotic rate in liver tissues in I-R group comparing to Sham group (Figure 1B).

Table II. TNF- α , IL-1 β and IL-6 contents at all time points after surgery.

Index	Group	6 h post-option	12 h post-option	24 h post-option
TNF- α (pg/ml)	Sham group	31.5±3.4	34.2±4.5	29.1±3.9
	I-R group	65.8±7.1*	106.5±10.7*	136.8±14.4*
IL-1 β (pg/ml)	Sham group	23.6±3.1	22.9±2.8	26.7±3.3
	I-R group	44.8±5.1*	68.7±5.9*	92.8±8.7*
IL-6 (pg/ml)	Sham group	51.1±6.6	48.7±7.1	53.8±7.6
	I-R group	88.3±7.3*	116.5±16.9*	149.7±21.6*

* $p < 0.05$ comparing to Sham group.

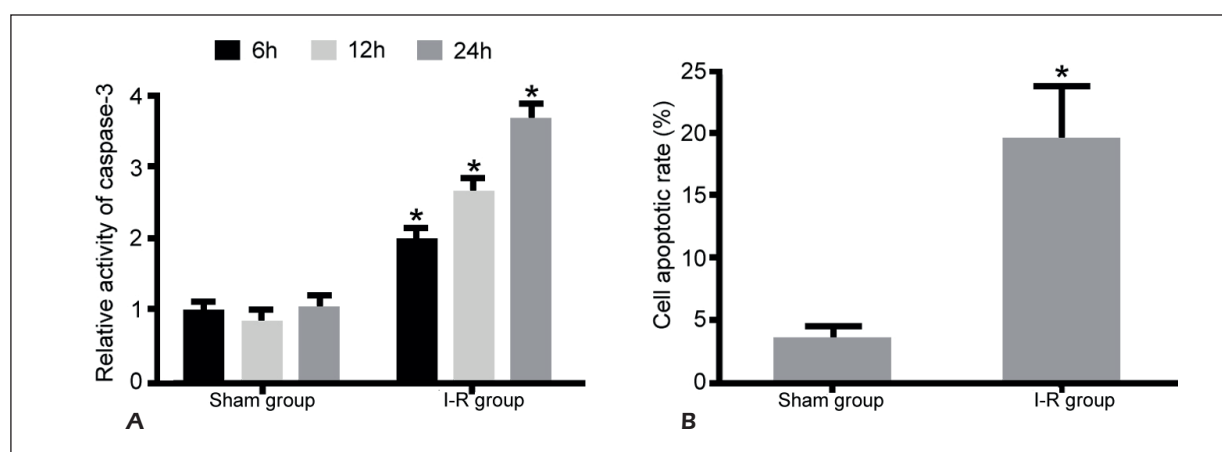


Figure 1. I-R challenge significantly induced liver cell apoptosis. **A**, Spectrometry for measuring caspase-3 activity in rat liver tissues. **B**, TUNEL assay for cell apoptosis in two groups of liver tissues. * $p < 0.05$ comparing to Sham group.

I-R Treatment Down-Regulated miR-93 Expression and Elevated STAT3 Expression in Liver Tissues

qRT-PCR results showed that, comparing to Sham group, I-R group liver tissues had significantly lower miR-93 expression. With elongated time, miR-93 expression was further decreased, whilst STAT3 mRNA level was remarkably elevated with correlation with I-R time (Figure 2A, B). Western blot results showed significantly higher STAT3 and p-STAT3 protein levels in liver tissues from I-R group rats comparing to Sham group (Figure 2C).

Targeted Regulatory Relationship Between miR-93 and STAT3

Online prediction on microRNA.org showed the existence of complementary binding sites between miR-93 and 3'-UTR of STAT3 mRNA (Figure 3A). Dual luciferase gene reporter assay showed that transfection of miR-93 mimic significantly depressed relative luciferase activity of HEK293T cells after transfecting pMIR-STAT3-wt, whilst having no effects relative luciferase activity of HEK293T cells transfecting with pMIR-STAT3-mut (Figure 3B), suggesting the targeted regulatory relationship between miR-93 and STAT3 mRNA.

Over-Expression of miR-93 Significantly Depressed I-R Induced Liver Damage and Alleviated Inflammatory Factor Release and Cell Apoptosis

Liver function assay showed that, comparing to agomir control group, miR-93 agomir group rats showed significantly lower serum ALT and

AST contents (Table III). ELISA results showed that intraperitoneal injection of miR-93 agomir significantly suppressed serum contents of inflammatory factors including TNF- α , IL-1 β , and IL-6 (Table III).

TUNEL results showed that, comparing to agomir control injection group, miR-93 agomir injection into the peritoneal cavity of I-R induced rats resulted into significantly lower hepatic cell apoptosis (Figure 4A), and suppressed caspase-3 activity in liver tissues (Figure 4B). Western blot results showed that comparing to agomir control group, miR-93 agomir rats showed significantly decreased STAT3 and p-STAT3 protein expression (Figure 4C).

Discussion

Current studies showed the involvement of multiple signaling pathways in hepatocyte I-R injury pathology, such as phosphatidylinositol-3-kinase/serine/threoninekinase (PI3K/AKT)¹³, mitogen-activated protein kinase (MAPK)¹⁴, and signal transducer and activator of transcription-Janus kinase (JAK-STAT)¹⁵. JAK-STAT signal transducing pathway can respond to various stimuli from extra-cellular growth factors and mitogen. Under these stimulating factors, membrane receptors undergo dimerization, which further phosphorylate and activate JAK kinase. Activated JAK kinase further phosphorylates receptor tyrosine, and facilitates the binding of STAT onto tyrosine phosphorylation site of receptor complex via SH2 domain. At this time, JAK kinase phosphorylates and activates STAT protein having spatial proximity.

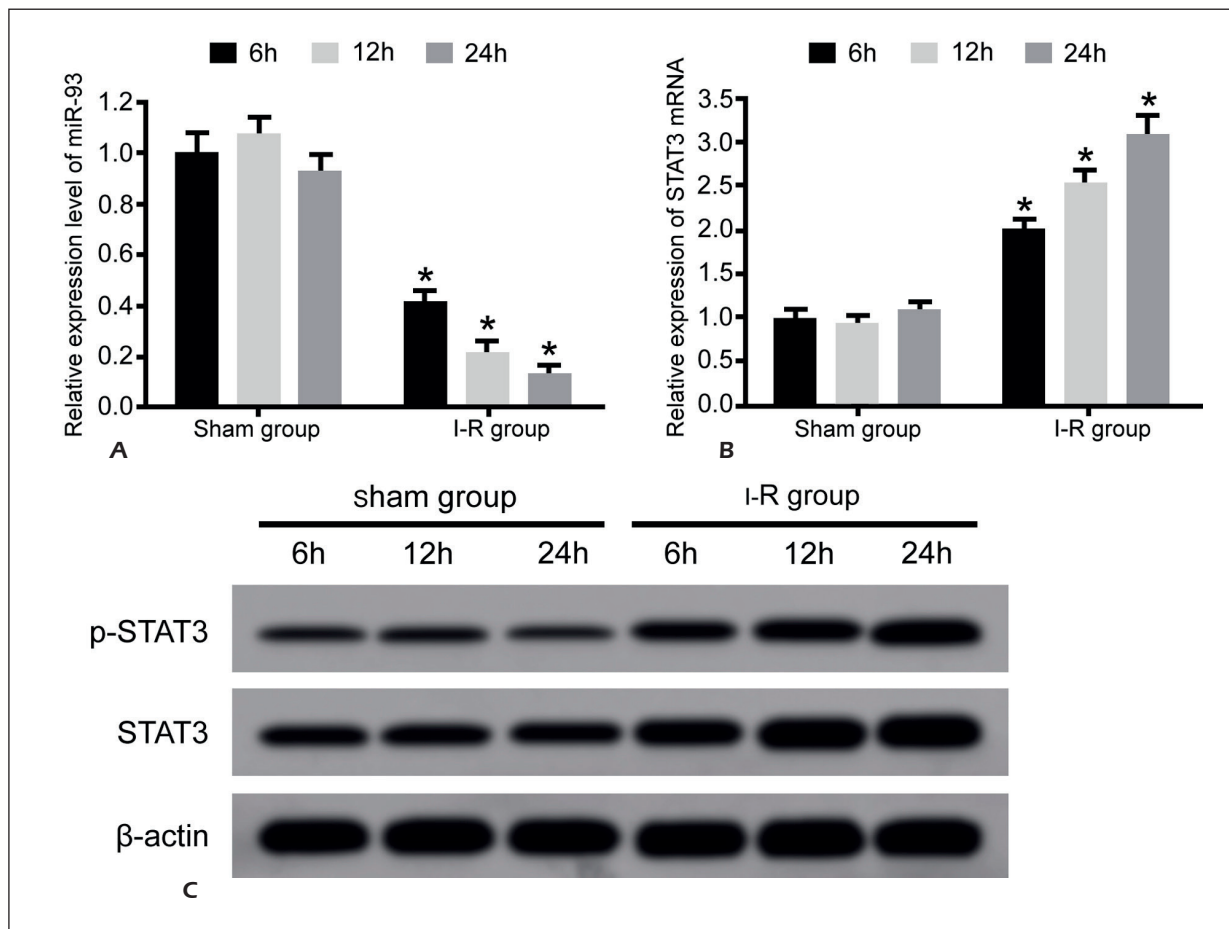


Figure 2. I-R treatment decreased liver miR-93 expression and increased STAT3 expression. **A**, qRT-PCR for miR-93 expression. **B**, qRT-PCR for STAT3 mRNA level in rat liver tissues. **C**, Western blot for liver protein expression. * $p < 0.05$ comparing to Sham group.

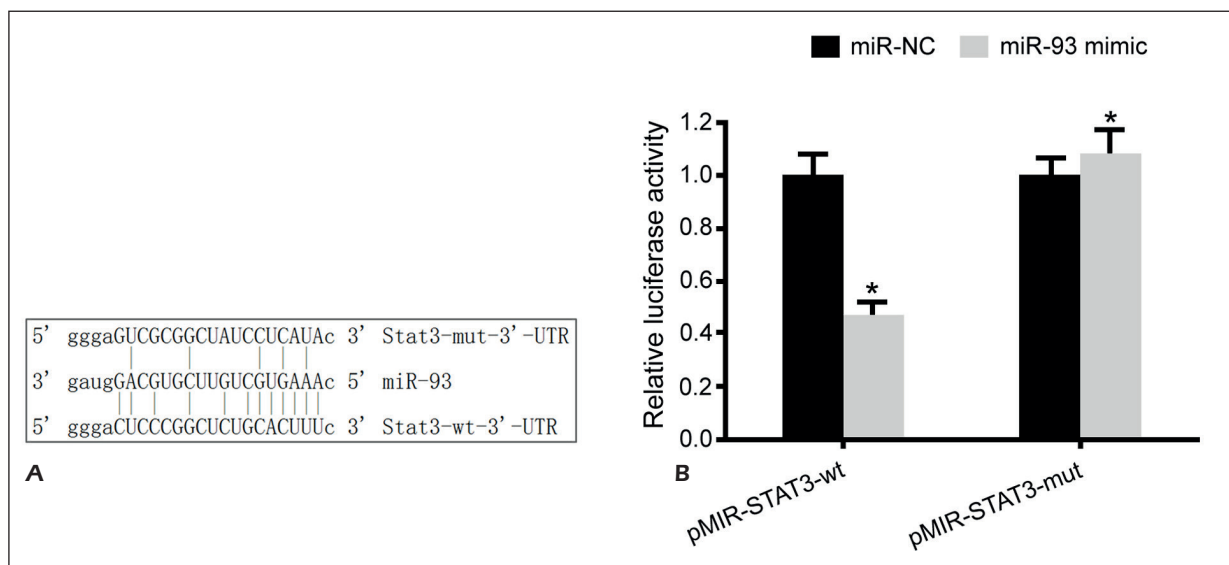
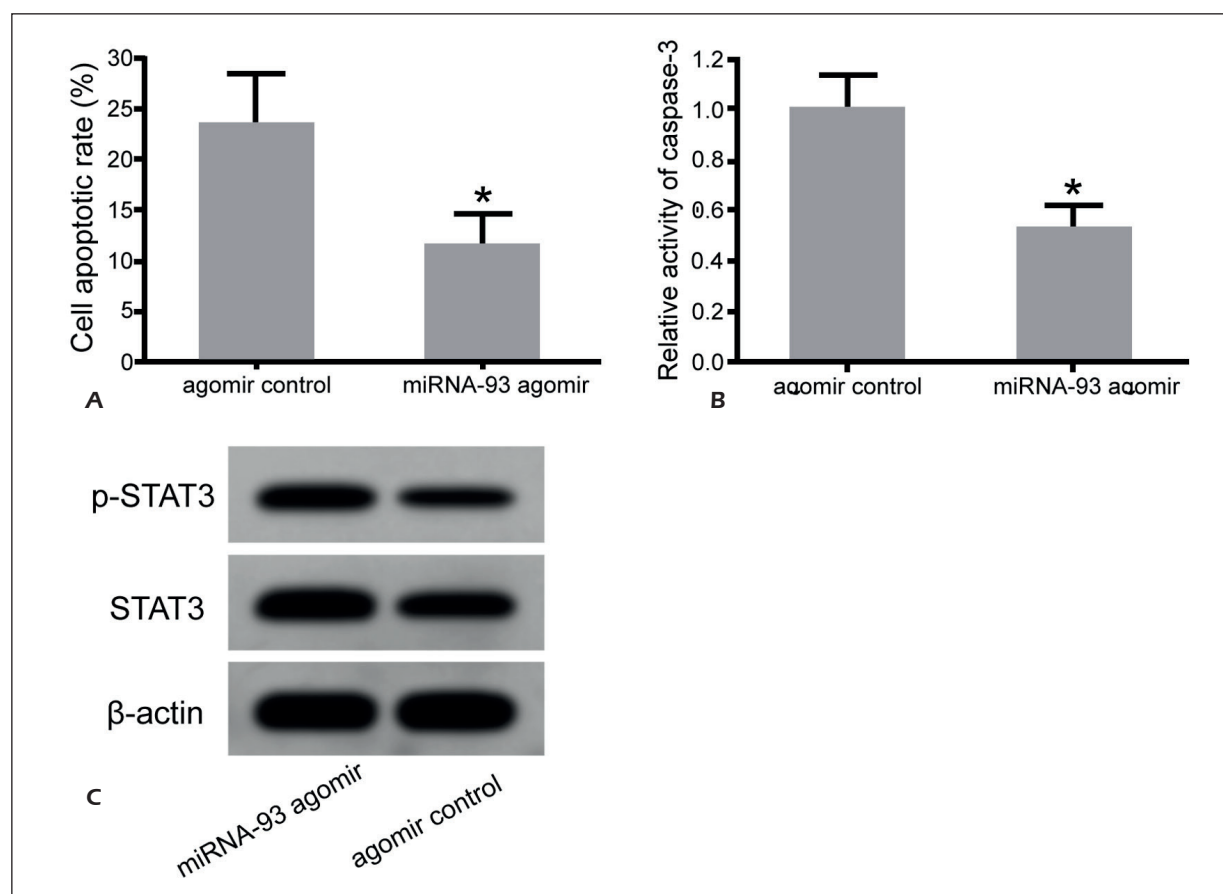


Figure 3. Targeted regulatory relationship between miR-93 and STAT3 mRNA. **A**, Binding sites between miR-93 and 3'-UTR of STAT3 mRNA. **B**, Dual luciferase gene reporter assay. * $p < 0.05$ comparing to miR-NC group.

Table III. Serum ALT, AST, TNF- α , IL-1 β and IL-6 levels at 24 h after surgery.

Group	ALT (U/L)	AST (U/L)	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-6 (pg/ml)
agomir control	383.9 $\pm$ 41.2	465.7 $\pm$ 59.4	124.5 $\pm$ 20.1	88.7 $\pm$ 11.4	133.8 $\pm$ 22.9
miR-93 agomir	201.5 $\pm$ 31.3*	294.8 $\pm$ 32.2*	81.5 $\pm$ 10.6*	41.7 $\pm$ 5.3*	55.9 $\pm$ 6.2*

* p <0.05 comparing to agomir control group.



Such activated STAT protein dissociates from receptor complex to form dimers and to facilitate trafficking of dimers from cytoplasm to nucleus, thus facilitating transcription and expression of genes related with cell proliferation, survival, and apoptosis¹⁶. JAK-STAT signaling pathway can modulate inflammatory response during pathological process of I-R injury^{7,17}, in addition to cell proliferation and apoptosis¹⁵, and is thus closely correlated with I-R injury pathogenesis^{18,19}. Several authors showed that multiple inflammatory factors including interferon- γ (IFN- γ), TNF- α ,

and IL-1 β could be regulated by JAK-STAT signal transducing pathway¹⁹⁻²¹, and exert direct or indirect roles in JAK-STAT induced I-R injury or inflammatory response¹⁹. STAT protein family consists of 7 members, including STAT1, STAT3, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Among those members, STAT3 is the most widely studied one, and its abnormal expression or function plays crucial roles in I-R injury of multiple tissues including heart⁴, brain⁵, and kidney⁶. Jia et al⁷ showed that during hepatic I-R injury, STAT3 expression and function were significantly po-

tentiated, indicating close correlation between STAT3 and liver I-R injury. MiR-93 belongs to miR-106b-25 gene family, and locates on chromosome 7q22.1. Researches^{11,12} showed that after I-R injury of heart or brain, miR-93 expression was significantly decreased, and miR-93 played important protective roles in I-R injury. However, whether miR-93 plays a regulatory role in liver I-R injury is still unclear.

This study showed that, compared to Sham group, I-R rats had significantly higher contents of ALT and AST in liver tissues, indicating liver dysfunction and hepatocyte damage, suggesting successful generation of I-R model. ELISA results found abnormally elevated serum levels of inflammatory factors including TNF- α , IL-1 β , and IL-6, suggesting the involvement of liver inflammation in liver I-R injury. TUNEL assay found that, compared to Sham group, I-R rats showed significantly enhanced hepatocyte apoptosis, indicating correlation between cell apoptosis and liver I-R injury. Rong et al² found significantly higher levels of liver and serum inflammatory factors including TNF- α , MCP-1, and IL-6 comparing to Sham group. Gendy et al³ showed that after I-R challenge, rat serum inflammatory factors TNF- α , and INF- γ were significantly up-regulated, accompanied with abnormally elevated caspase-3 activity in liver tissues. Sadatomo et al²² showed that, during liver I-R injury, inflammatory cells including neutrophil and macrophage could interact to facilitate production of inflammatory factor IL-1 β in liver tissues. The production of IL-1 β is critical for inducing liver I-R injury. The role of various macrophages in liver I-R injury has been demonstrated by various studies. During early stage of liver I-R injury, liver tissues showed abundantly activated macrophage, which led to cascade inflammatory response to release inflammatory factors causing major apoptosis of hepatocytes. Apoptosis is the important form of hepatocyte death, eventually leading to liver dysfunction²³. In this investigation, we found prominent hepatocyte apoptosis in I-R rats, along with potentiated inflammatory response, as similar with Rong et al², Gendy et al³, and Sadatomo et al²².

Reports showed abnormal STAT3 expression or activity after I-R injury of multiple tissues/organs including heart⁴, brain⁵, and kidney⁶, indicating correlation between STAT3 and I-R injury. Freitas et al¹⁵ found significantly enhanced phosphorylation and function of STAT3 after liver I-R. Athanasopoulos et al²⁴ also showed

significantly elevated STAT3 expression in liver I-R injury. Moreover, Han et al²⁵ observed significantly elevated STAT3 expression during liver I-R injury and hypothesized that STAT3 up-regulation was one critical molecular event of liver I-R injury. We found significantly elevated expression of STAT3 and p-STAT3 after rat liver I-R injury, agreeing with Freitas et al¹⁵, Athanasopoulos et al²⁴, and Han et al²⁵. Several studies found close relationship between miR-93 and I-R injury. Hazarika et al²⁶ found that over-expression of miR-93 significantly relieved I-R injury of bone muscle cells and vascular endothelial cells by decreasing apoptosis. They also found that injection of pre-miR-93 remarkably improved I-R injury of mouse hindlimb, whilst antagomir-93 injection significantly inhibited I-R injury recovery of mouse hindlimb. Ke et al¹² found significantly lower miR-93 expression in rat cardiomyocytes with I-R challenge, and transfection of miR-93 mimic significantly decreased I-R induced rat cardiomyocyte apoptosis and relieved oxidative stress injury. Li et al²⁷ showed that miR-93 up-regulation after myocardial infarction could help to protect cardiomyocytes from I-R induced apoptosis. Tian et al¹¹ showed lower miR-93 expression in mouse brain after cerebral I/R, and injection of miR-93 mimic ago-miR-93 into mouse shrank cerebral infarction volume, improved neurological function, and decreased brain inflammatory response and cell apoptosis rate. Similar results were obtained in this study, as I-R rat showed significantly decreased miR-93 expression in liver tissues, and the correlation between miR-93 level and I-R time. Bioinformatics analysis revealed the targeted complementary binding sites between miR-93 and 3'UTR of STAT3 mRNA, indicating possible targeted relationship between them. We used dual luciferase gene reporter assay to demonstrate that miR-93 could regulate STAT3 expression. Freitas et al¹⁵ showed that, after using JAK2-STAT3 pathway antagonist AG490 to suppress STAT3 functional activity, the hepatic injury, cell apoptosis, inflammatory cell infiltration, and release of inflammatory factors TNF- α , IL-1 β and IL-6 were all alleviated. After depressing STAT3 expression or functional activity, we found that liver I-R injury, cell apoptosis, and inflammatory factor release were all significantly inhibited, as consistent with Freitas et al¹⁵. This research found the role of miR-93 down-regulation in inducing STAT3 up-regulation and in facilitating liver I-R injury, inflamma-

tion and apoptosis, and revealed the potential role of miR-93 over-expression in suppressing STAT3 expression and in alleviating liver I-R injury. So far various studies have reported the relationship between miR-93 and I-R injury, but are largely limited in cardiomyopathy or brain damage, leaving liver I-R injury unattended. This study for the first time revealed the role of miR-93 in liver I/R injury. However, whether similar effects of miR-93 in regulating liver I-R injury in human population via modulating STAT3 still remains unclear, and requires further investigation, thus making the weakness of the current work.

Conclusions

We found that in liver I-R injury, miR-93 was down-regulated whilst STAT3 expression was significantly elevated. Overexpression of miR-93 could remarkably depress STAT3 expression in liver I-R injury, alleviate hepatic damage and cell apoptosis, decreased inflammatory response, and improved liver function.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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