hsa-miR-34a-5p Ameliorates Hepatic Ischemia/Reperfusion Injury Via Targeting HNF4α

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ABSTRACT

Background: *To investigate the relationship between the expression level of hsa-miR-34a-5p and liver injury and to further explore its regulatory signaling pathways*

Methods: *Liver tissue and blood were collected from 60 patients undergoing hepatectomy. We constructed a rat HIRI model and treated it with an intraperitoneal injection of agomir-miR-34a-5p or agomir-normal control (NC) for 7 days after the surgery. The pathological changes of agomir-miR-34a-5p or agomir-normal control (NC) groups were compared. 7702 and AML12 cells were transfected with mimics NC or miR-34a-5p mimics and then treated with H_2O_2 for 6 hours. Cell apoptosis was detected by flow cytometry, Western blot, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, respectively. Furthermore, the target genes of miR-34a-5p were identified by luciferase reporter gene assay and were verified in vitro.*

Results: *The relatively high miR-34a-5p expression group revealed a lower level of alanine aminotransferase and aspartate aminotransferase compared with the relatively low miR-34a-5p expression group. HIRI+agomir-miR-34a-5p rats exhibited significantly higher miR-34a-5p expression, lower serum alanine aminotransferase, aspartate aminotransferase, alleviated hepatic necrosis, reduced hepatocyte apoptosis, and decreased expression of apoptosis-related proteins, when compared with HIRI+agomir-NC rats ($P < .05$). After hydrogen peroxide treatment, alpha mouse liver-12 cell (AML-12) and normal liver cell line LO2 (LO2) cells transfected with miR-34a-5p mimics had significantly lower apoptosis rate compared with miR-34a-5p mimics NC group ($P < .05$). Hepatocyte nuclear factor 4α was identified as a miR-34a-5p target gene. Hepatocyte nuclear factor 4α expression was significantly downregulated in AML12 and HL-7702 (7702) cells transfected with miR-34a-5p ($P < .05$). Moreover, AML12 and 770_2 cells transfected with miR-34a-5p significantly showed higher c-Jun N-terminal kinase (JNK), P38, cleavage cas-3, and BCL2 associated X (Bax) protein levels compared with AML12 and 7702 cells transfected with agomir-NC.*

Conclusion: *miR-34a-5p possibly protected the liver from I/R injury through downregulating Hepatocyte nuclear factor 4α to inhibit the JNK/P38 signaling pathway.*

Keywords: *hsa-miR-34a-5p, hepatic, HNF4α, ischemia/reperfusion injury, JNK/P38 signaling pathway*

INTRODUCTION

Hepatic I/R injury is a common pathological process after hepatic blood flow occlusion.[1,2] Long-term ischemia–reperfusion leads to decreased residual liver recovery ability, prolonged recovery time, and even liver failure and patient death. A study has shown that 10% of patients undergoing liver transplantation had postoperative liver dysfunction, biliary complications, and nonfunctioning liver function, which were associated with ischemia–reperfusion injury.[3] Large numbers of reactive oxygen species generated, Kupffer cell activated, neutrophil aggregated, and increased inflammatory cytokines in hepatic I/R injury can cause hepatic endoplasmic reticulum destruction, intracellular calcium overload, mitochondrial abnormalities, and ultimately hepatocyte apoptosis and necrosis.[4-6]

MicroRNA (miRNA) is involved in cell differentiation, proliferation, metabolism, hemostasis, apoptosis, and inflammation.[7-11] Previous studies have shown that miRNAs such as miR-122, miR-34a, miRNA-29c, miRNA-182, and miRNA-190 have vital effects in I/R injury.[12-14] The expression of miR-34a-5p is upregulated after myocardial injury, and inhibition of miR-34a-5p expression can protect myocardial ischemia–reperfusion injury.[15,16] However, the role of miR-34a-5p in liver I/R injury remains unclear. In this study, we demonstrated the correlation between miR-34a-5p and I/R injury through collecting and testing

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liver samples of clinical patients. We also explored the specific function of miR-34a-5p and its potential signal pathway in hepatic I/R injury rats and hepatic AML12 and 7702 cells treated with hydrogen peroxide (H_2O_2)-induced stress. Our study may provide novel insights into the pathology and therapeutic to I/R injury.

MATERIALS AND METHODS

Clinical Sample Collection

Liver tissue and blood were collected from 60 hepatocellular carcinoma patients undergoing hepatectomy 2019 and 2020. The time of porta hepatis occlusion was 20 minutes in all 60 cases of hepatectomy. Therefore, the 60 cases of liver have the same ischemia–reperfusion injury. Samples were frozen in liquid nitrogen until RNA extraction. After blood separation, the supernatant was stored at -80°C until transaminases were detected. All patients received written informed consent before surgery. This study was authorized by the hospital's ethics committee. All research methods followed the Declaration of Helsinki and conformed to the principles of medical ethics.

Quantitative Real-Time PCR

Total RNA from frozen liver tissues was extracted with Trizol (Invitrogen, Carlsbad, Calif, USA) based on the manufacturer's protocol. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on an ABI Prism 7500 using SYBR Green PCR Master Mix (Takapa, Qingdao, China). The primers used in the PCR were as follows: miR-34a-5p; β-actin. miR-34a-5p RNA expression was calculated using the $2^{-\triangle\triangle CT}$ method, and β-actin was used to be normalized. The sequences of the primers are shown in Table 1.

Main Points

- *hsa-miR-34a-5p is associated with milder liver injury in adults after hepatectomy.*
- *hsa-miR-34a-5p attenuated hepatic I/R injury in rats.*
- *hsa-miR-34a-5p decreased the level of apoptosis in liver I/R injury and reduced the hydrogen peroxide-induced apoptosis of hepatic AML12 and 7702cells by inhibiting the JNK/P38 in vitro.*
- *Hepatocyte nuclear factor 4α (HNF4α) is identified as a target gene of miR-34a-5p.*
- *Hsa-miR-34a-5p attenuated hepatic I/R injury via targeting HNF4α.*

Table 1. Primers Sequences

Name	Sequences
Primers for real-time PCR	
miR-34a-5p forward primer	5′-CTGGGAGGTGGCAGTGTCTTAGC-3′
miR-34a-5p reverse primer	5′-TCAACTGGTGTCGTGGAGTCGG-3′
HNF4α forward primer	5′-CACGGGCAAACACTACGGT-3′
HNF4α reverse primer	5′-TTGACCTTCGAGTGCTGATCC-3′
JNK forward primer	5′-GGGTATGCCCAAGAGGACAGA-3′
JNK reverse primer	5′-GTGTTGGAAAAGTGCGCTGG-3′
P38 forward primer	5′-AACATCCTGTCGTCGCCTTAC-3′
P38 reverse primer	5′-ACGTGCGTGACCTTAAAGTAGA-3′
Cas-3 forward primer	5′-CATGGAAGCGAATCAATGGACT-3′
Cas-3 reverse primer	5′-CTGTACCAGACCGAGATGTCA-3′
Bax forward primer	5′-CATATAACCCCGTCAACGCAG-3′
Bax reverse primer	5′-GCAGCCGCCACAAACATAC-3′
GAPDH forward primer	5′-TGTGGGCATCAATGGATTTGG-3′
GAPDH reverse primer	5′-ACACCATGTATTCCGGGTCAAT-3′

Liver Enzyme Assay

Using kits (Shanghai Future Industrial Co, Shanghai, China) to detect serum alanine aminotransferase (ALT), aspartate transaminase (AST), oxidative parameters of the liver including MDA, TOA, and OSI to assess liver damage following the manufacturer's instruction.

Construction of a Rat HI/RI Model

Male Sprague–Dawley (SD) rats (10-12 weeks old) were purchased from Shanghai Jiake Biotechnology Co, Shanghai, China. All animals are maintained in specific pathogen free (SPF) animal rooms. The animal management committee of the First Affiliated Hospital of Nanchang University approved the study. Non-invasive arterial clamps were used to clamp arteries and portal veins supply to the middle and left hepatic to make a model of hepatic ischemia (70%). After 1 hour of ischemia, releasing the clamps and reperfusing the liver for 6 hours before sacrifice. SD rats were randomly divided into HIRI+agomir-NC, HIRI+agomir-miR-34a-5p groups (n = 12, each groups). Rat in HIRI+agomir-NC and HIRI+agomir-miR-34a-5p groups respectively were injected 20 μL of 500 pmol agomir-NC and agomir-miR-34a-5p (Shanghai Jikai Biotechnology Co., Ltd). Blood

and liver samples were collected when the surgery was finished.

Histological Examination

The rat ischemic-reperfusion liver tissue was fixed in 4% buffered formalin and embedded in paraffin and cut into 5 μm thick sections. Hepatic sections were stained with Hematoxylin and Eosin (HE), and pathological changes such as inflammatory infiltration and hepatocyte necrosis were observed under light microscope.

Apoptosis Assay

Apoptosis cells of liver tissues were tested by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (16211 N.E. 44TH CT. Redmond, Washington, United States). TUNEL positive cells were calculated in 5 randomly selected sections.

Immunofluorescent Staining

Paraffin sections were deparaffinized with xylene, and endogenous peroxidase vitality was inactivated with 3% H_2O_2, blocked with 5% bovine serum albumin for 30 minutes. The slides were incubated with anti-caspase-3 antibody (Pudong New Area, Shanghai), anti-BAX antibody (Abcam), anti-JNK antibody (Abcam, USA), anti-P38 antibody (Abcam), at 4°C overnight. Then, The slides were incubated with biotin-labeled secondary antibody immunoglobulin (Xicheng District, Beijing, China) and horseradish peroxidase-labeled streptavidin for 60 minutes at 37°C. The 3,3-N-Diaminobenzidine Tertrahydrochloride (DAB) was used to counter-stain the nuclei for 10 minutes. The slides were observed on fluorescence microscope, and yellow or brown stained cells are positive cells.

Western Blot Analysis

Total protein was extracted from harvested liver tissue or cells using lysis buffer (Beyotime, Shanghai, China) and quantified using the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime) according to the manufacturer's instructions. The protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and transferred to a poly vinylidene fluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with 5% skim milk and incubated overnight at 4°C with a suitably diluted primary antibody as follows: cleavage cas-3, Bax, JNK, P38, hepatocyte nuclear factor 4α (HNF4α), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The membrane was then washed with phosphate-buffered saline with Tween 20 (PBST) and incubated with (secondary antibody) for 1 hour at room temperature. Immunoreactivity was detected using the Electrochemiluminescence (ECL) detection system. All antibodies were purchased from Abcam (Cambridge, Mass, USA). GAPDH was used as an internal control.

Cell Culture and Transfection

Human hepatic cell line 7702 and murine hepatic cell line AML12 cells were cultured from Cell bank of Shanghai Chinese Academy of Sciences. 7702 and AML12 cells at 70% confluence in 6-well plates were transfected with 50 nM of miR-494 mimics, mimics NC using Lipofectamine 3000 transfection agent. After 48 hours, total RNA and protein were extracted, and HNF4α mRNA and protein expression were analyzed by qRT-PCR and by Western blot (WB) as described above.

Apoptosis Detection

Transfected 7702 cells and AML12 cells were treated with 200 μm H_2O_2 for 6 hours, and then, the cells were harvested and resuspended in 500 μL of binding buffer. Annexin V-FITC (5 μL) was added, and apoptosis was detected by flow cytometry within 30 minutes. At the same time, TUNEL assay was used to detect the apoptosis level of the 2 groups, and the expression of cleavage cas-3, Bax, JNK, P38, and HNF4α was detected by WB.

Prediction of miR-34a-5p Targets

Potential downstream target genes of miR-34a-5p were identified using TargetScan version 6.2. The effect of overexpression of miR-34a-5p on the gene expression was detected by RNA-Seq. The genes predicted by these 2 databases are considered to be potential target genes for miR-34a-5p. The HNF4α target was selected for further analysis based on the score of the TargetScan. The bioinformatics method analyzes miR-34a-5p-regulated signaling pathways associated with ischemia–reperfusion injury.

Luciferase Reporter Assay

The HNF4α gene was analyzed using an online tool to predict the 3-UTR sequence of miR-34a-5p. The oligonucleotide sequences (HNF4α wild type and mutant 3-UTR) were cloned into the pMIR-Reporter (pMIR) (Hollow Road, Madison, Wisconsin, USA) site of the firefly luciferase

reporter vector.7702 cells and AML12 cells were cotransfected at 70% confluence with 500 ng pMIR—HNF4α-wt/pMIR—HNF4α- mut and 50 nM miR-34a-5p mimic/mimetic C, (No. 1011 Halley Road, Zhangjiang Hi-Tech Park, Shanghai, China) using Lipofectamine 3000 transfection kit (Invitrogen). After 24 hours, luciferase activity was determined using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Detection of miR-34a-5p Protecting Hepatic I/R Mechanism

The 7702 and AML12 cell lines were each divided into 4 groups, which were infected with flag-HNF4α+agomir-NC, agomir-NC group, flag-HNF4α+agomir-miR-34a-5p, and agomir-miR-34a-5p, using Lipofectamine 3000 transfection agent and employed equal amount of H_2O_2 treatment. TUNEL assay was used to detect the apoptosis level of the each groups, and the expression of cleavage cas-3, Bax, JNK, P38, and HNF4α was detected by WB.

Statistical Analysis

All experiments were repeated 3 times. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 19.0 software (SPSS Inc.; Chicago, IL, USA). Differences between the 2 groups were determined by the *t*-test. Univariate analysis of variance and Tukey HSD test were used to compare the differences between groups, $P < .05$ was considered statistically significant.

RESULTS

hsa-miR-34a-5p is Associated with Milder Liver Injury in Adults After Hepatectomy

To explore the relationship between the expression level of hsa-miR-34a-5p and liver injury, liver tissue and blood samples from 60 patients undergoing hepatectomy were collected. The expression level of hsa-miR-34a-5p in every liver sample was detected, and 60 samples were divided equally into 2 groups (high miR-34a-5p and low miR-34a-5p expression). As shown, the relatively high miR-34a-5p expression group revealed a lower level of ALT and AST compared with the relatively low miR-34a-5p expression group (Figure 1A). In the correlation analysis, there is a certain negative correlation between the expression of hsa-miR-34a-5p and ALT, AST, respectively (Figure1 B–C). The above results suggested that high expression of hsa-miR-34a-5p may cause less liver injury in hepatectomy of adults.

hsa-miR-34a-5p Attenuated Hepatic I/R Injury in Rats

To explore the role of hsa-miR-34a-5p in I/R injury, we treated the HIRI rats with an intraperitoneal injection of agomir-hsa-miR-34a-5p or agomir-NC. qRT-PCR results showed that the expression of hsa-miR-34a-5p in the rats injected with agomir-hsa-miR-34a-5p was significantly higher than that in the rats injected with agomir-NC (Figure 2A). Less liver edema and inflammatory cell infiltration were seen in the group with high expression of miR-34-a-5p (Figure 2B). Meanwhile, oxidative parameters of the liver including MDA, TOA, and OSI reduced significantly in HIRI+ agomir-hsa-miR-34a-5p group compared with

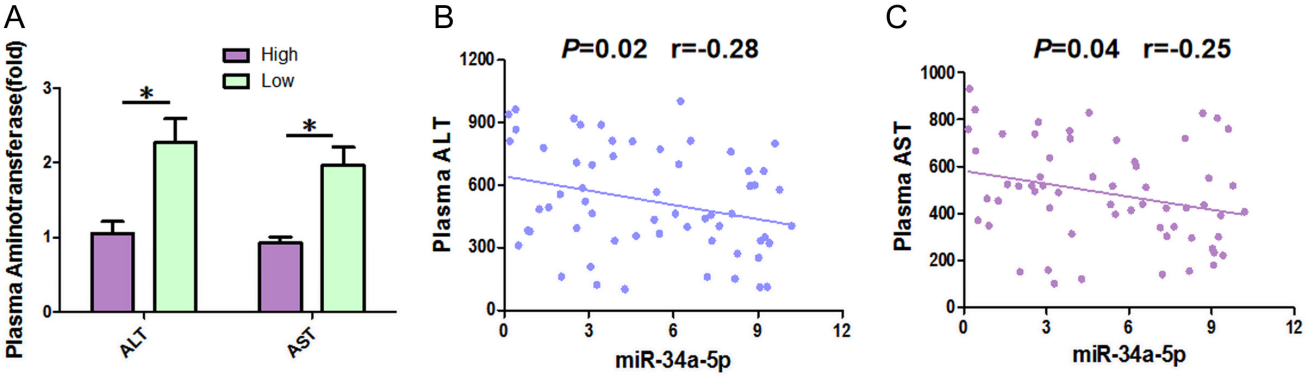

Figure 1. Hsa-miR-34a-5p is associated with milder liver injury in adults after hepatectomy postoperative. Blood samples were collected from 60 patients with hepatectomy. qRT-PCR was used to detect the expression of hsa-miR-34a-5p, and the chemiluminescence method was used to detect the expression of transaminase ALT and AST in blood. (A) The expression of transaminase ALT and AST in blood was detected. The expression level of transaminase in the relatively high miR-34a-5p expression group was lower. (B) There is a certain negative correlation between miR-34a-5p and ALT, r = −0.28, $P < .05$. (C) There is a certain negative correlation between miR-34a-5p and AST, r = −0.25, $P < .05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

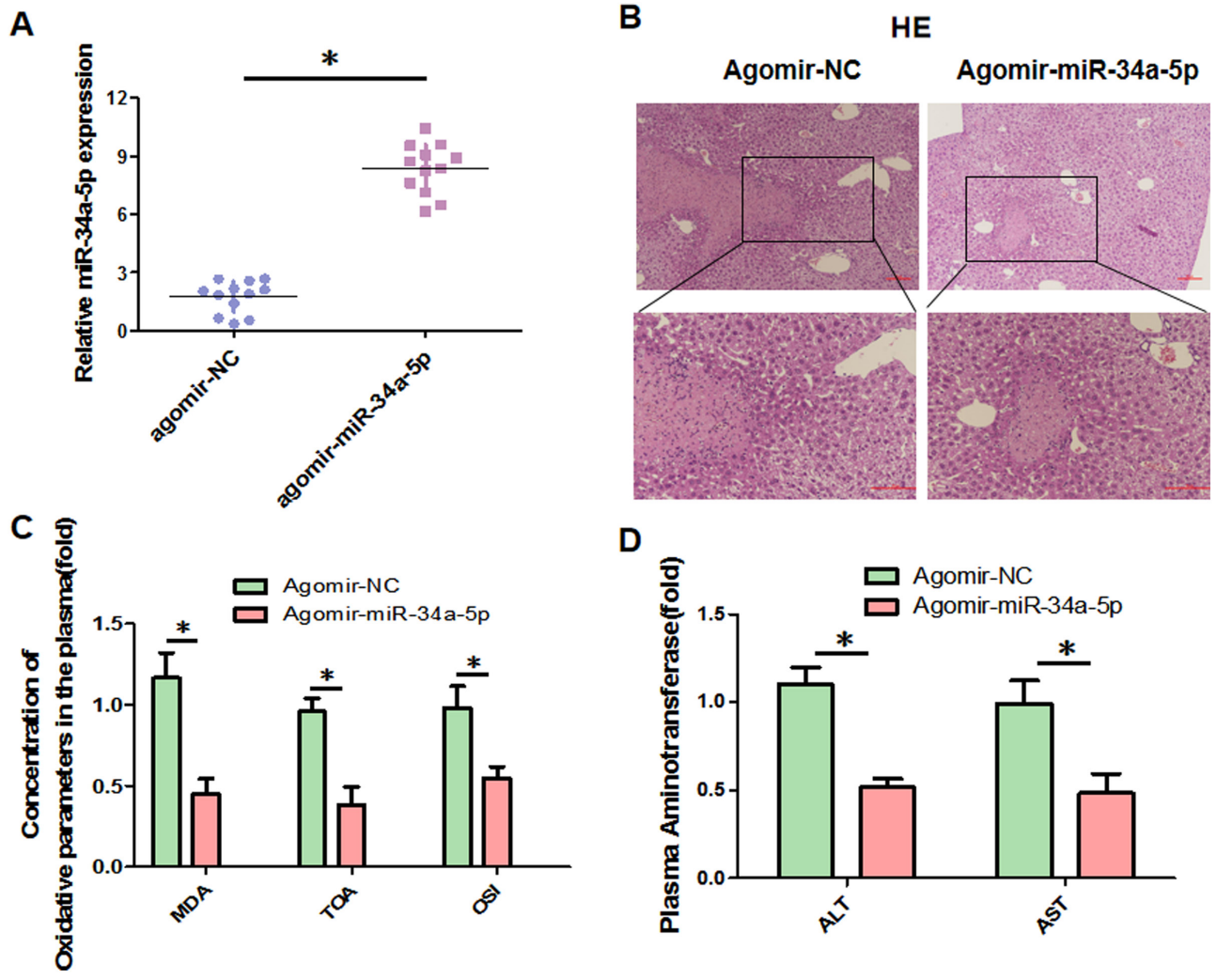

Figure 2. hsa-miR-34a-5p attenuated hepatic I/R injury in rats. HIRI rats (n = 12 rats per group) were treated with agomir-*miR-34a-5p* or agomir-NC. (A) *miR-34a-5p* expression level in liver samples from different groups was measured by qRT-PCR. (B) HE staining of liver samples. Arrow indicates hepatocyte injury. (C) Concentration of liver oxidative parameters MDA, TOA, and OSI. (D) Serum level of ALT and AST was determined using commercial kits. All experiments were performed in triplicate, and data were represented as mean ± S.D. $^{*}P < .05$ compared with sham group. qRT-PCR, quantitative real-time polymerase chain reaction; ALT, alanine aminotransferase; AST, aspartate aminotransferase

HIRI+ agomir-NC group (Figure 2C), demonstrating that miR-34a-5p can curb the production of oxidative stress. Moreover, there was a lower level of ALT and AST in the miR-34a-5p group, which revealed that miR-34a-5p could attenuate hepatic I/R injury (Figure 2D).

hsa-miR-34a-5p Decreased the Level of Apoptosis in Liver I/R Injury and Reduced the H_2O_2-Induced Apoptosis of Hepatic AML12 and 7702 Cells by Inhibiting the JNK/P38 In Vitro

In order to investigate how miR-34a-5p affects I/R injury, we performed TUNEL assay, immunohistochemistry (IHC), and Western blot (WB) assays on rat liver specimens. TUNEL assay showed that miR-34a-5p group had a lower number of TUNEL-positive cells than sham group (Figure 3A). IHC results suggested high expressions of cleavage cas-3, Bax, JNK, and P38 in sham group (Figure 3B). Further, we also found high expressions of those proteins including cleavage cas-3, Bax, JNK, and P38 by WB (Figure 3C). All together, these results indicated that overexpression of miR-34a-5p inhibited the apoptosis of liver cells of rats, as well as apoptosis-related signaling pathways and apoptosis-related proteins. To validate our conclusion, these tests were also performed on 2 cell lines, 7702 cell line and

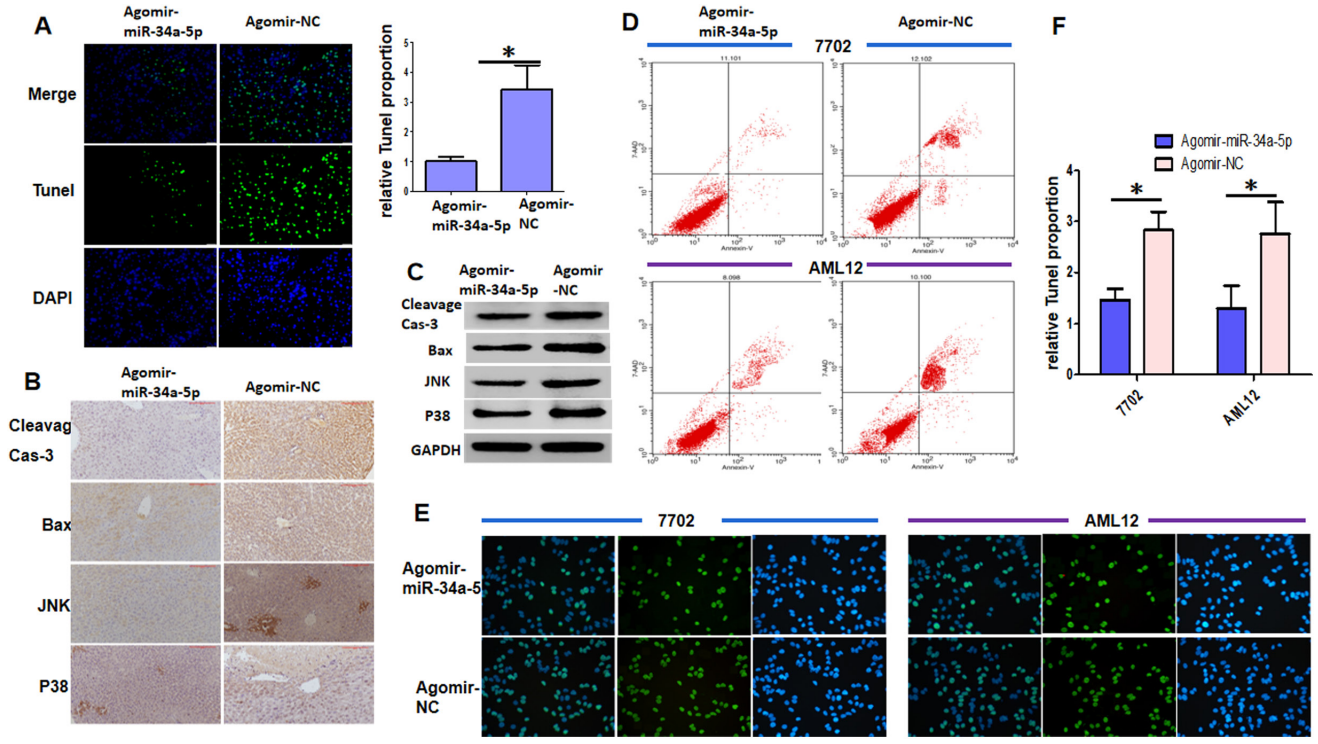

Figure 3. hsa-miR-34a-5p decreased apoptosis proteins expression in liver I/R injury and protected hepatic AML12 and 7702 cells from H_2O_2-induced damage by inhibiting the JNK/P38 (A) TUNEL staining of hepatocellular apoptosis. (B) IHC detection of JNK, P38, Bax, and cleaved caspase-3 in different groups. (C) Western blot detection of JNK, P38, Bax, cleaved caspase-3 in different groups using GADPH as an internal control (graphs with error bars in Supplementary Figure 1). (D) Cell apoptosis was detected by flow cytometry (annexin V-FITC staining) in hepatic 7702 and AML2 cells treated by H_2O_2. *$P < .05$ compared with mimics NC group. (E) TUNEL staining of hepatocellular apoptosis in hepatic 7702 and AML2 cells treated by H_2O_2. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

AML12 cell line. Both cell lines were transfected with miR-34a-5p mimics or mimics NC before being treated with 200 μM H_2O_2 for 6 hours. Under the observation of flow cytometry, the number of apoptotic cells is more in mimics NC group than in miR-34a-5p mimics group Figure 3D. Meanwhile, more TUNEL-positive cells could be found in mimics NC group (Figure 3E–F). Thus, overexpression of miR-34a-5p can attenuate H_2O_2-induced apoptosis of hepatocytes.

HNF4α is Identified as a Target Gene of miR-34a-5p

To search for the target gene of miR-34a-5p, the prediction and scoring were performed through TargetScan website; RNA-Seq detected the changes of gene expression in liver cells after overexpression of miR-34a-5p. miR-34a-5p's target genes are supposed to be the intersection of the 2 sets of data, so we drew a heat map based on the 2 sets of data (Figure 4A). Further bioinformatics analyses showed HNF4α was identified as one of the candidate of miR-34a-5p and involved in JNK, P38 signals regulations (Figure 4B). 7702 and AML12 cells were treated with agomir-miR-34a-5p or agomir-NC as described above. In the detection of mRNA expression by qRT-PCR, it was found that the expression of HNF4α was lower in the cells treated with agomir-miRNA-34a-5p compared with the cells treated with agomir-NC (Figure 4C). Further, WB suggested that HNF4α protein was downregulated in agomir-miR-34a-5p groups (Figure 4D). In the Luciferase Report assay, the level of promoter luciferase reporter gene plasmid was constructed and found that it was also lower in agomir-miR-34a-5p groups (Figure 4E–F). All these findings elucidated that overexpressed miR-34a-5p inhibited HNF4α mRNA and protein and promoter luciferase reporter gene plasmid levels in hepatocyte cells. To confirm its binding sites, we predicted the binding sites through professional websites (http://www.targetscan) and verified them by the following experiments (Figure 4E). Promoters luciferase reporter gene plasmids for a mutant binding site (mut group) were also constructed in hepatocyte cells (Figure 4E). The luciferase reporter assay showed that

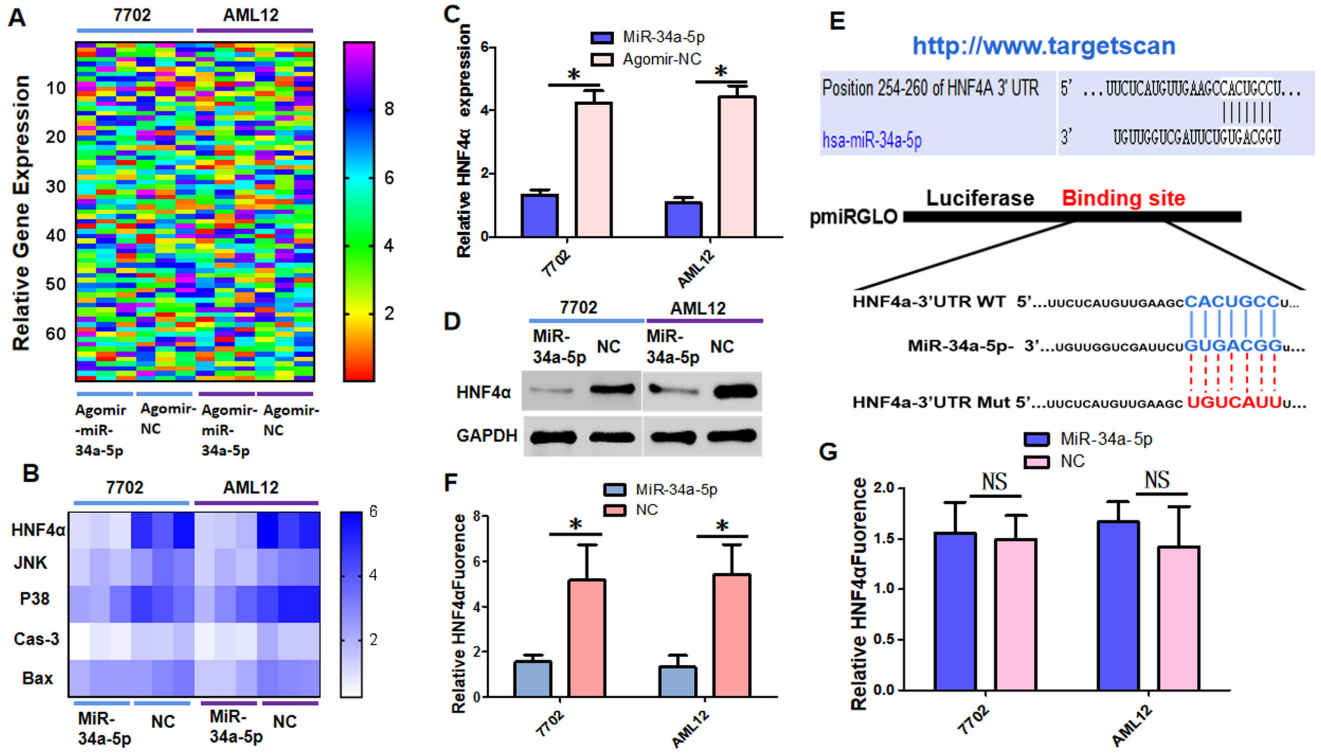

Figure 4. HNF4α is identified as a target gene of miR-34a-5p. (A) A large number of potential target genes for miR-34a-5p have been predicted from TargetScan sites, including HNF4α. Color bar represents the relative level of gene expression detected by RNA-Seq. (B) Bioinformatics analyses to find whether HNF4α with highest score was identified as one of the candidate of miR-34a-5p and involved in JNK and P38 signals regulations or not. Color bar represents the relative level of gene expression. (C and D) 7702 and AML12 cells were transfected with a miRNA-34a-5p mimics, mimic NC. The expression of HNF4α mRNA and protein was detected by qRT-PCR and Western blot (Graphs with error bars in Supplementary Figure 1), respectively. All experiments were performed in triplicate, and data were represented as mean + S.D. $^{**}P < .01$ compared with mimic NC. (E) Find HNF4α binding sites by searching http://www.targetscan. Promoter luciferase reporter plasmid was successfully constructed with mutant binding sites (mut group) and wild type binding site (wt group) in 7702 and AML12 cells. (F) The expression of HNF4α promoter luciferase reporter gene plasmid was detected in agomir-miRNA-34a-5p and agomir- NC groups. (G) The expression of HNF4α promoter luciferase reporter gene plasmid with mutant binding sites was detected in agomir-miRNA-34a-5p and agomir- NC groups. All experiments were performed in triplicate, and data were represented as mean ± S.D. $^{*}P < .05$ compared with sham group. HNF4α, hepatocyte nuclear factor 4α; qRT-PCR, quantitative real-time polymerase chain reaction.

miR-34a-5p had no impacts in mut group (Figure 4G). The results together confirmed that HNF4α is a downstream target gene of miR-34a-5p.

Hsa-miR-34a-5p Attenuated Hepatic I/R Injury via Targeting HNF4α

To further explore the molecular mechanism of miR-34a-5p attenuating hepatic ischemia–reperfusion injury, we conducted the experimental study on 7702 and AML12 cells, respectively. They were divided into 4 groups: flag-HNF4α+agomir-NC group, agomir-NC group, flag-HNF4α+agomir-miR-34a-5p group, and agomir-miR-34a-5p group dealing with an equal amount of H_2O_2. TUNEL experiments showed that the apoptosis hepatic cell of flag-HNF4α+agomir-NC group was the highest, and the apoptosis rate was the lowest in the agomir-miR-34a-5p group, while the apoptosis hepatic cell of flag-HNF4α+agomir-miR-34a-5p group was between flag-HNF4α+agomir-NC and agomir-miR-34a-5p group, indicating that HNF4α promoted hepatocyte apoptosis and miR-34a-5p inhibited hepatocyte apoptosis possibly through HNF4α (Figure 5A). Western blot results showed that compared with the flag-HNF4α+agomir-miR-34a-5p and agomir-NC groups, the expression of HNF4α of agomir-miR-34a-5p group was significantly inhibited, and the expression levels of apoptosis-related proteins such as JNK, P38, cas-3, and Bax were also significantly decreased, indicating that miR-34a-5p downregulates HNF4α and inhibits the expression of various apoptotic factors to protect the liver from I/R injury (Figure 5B).

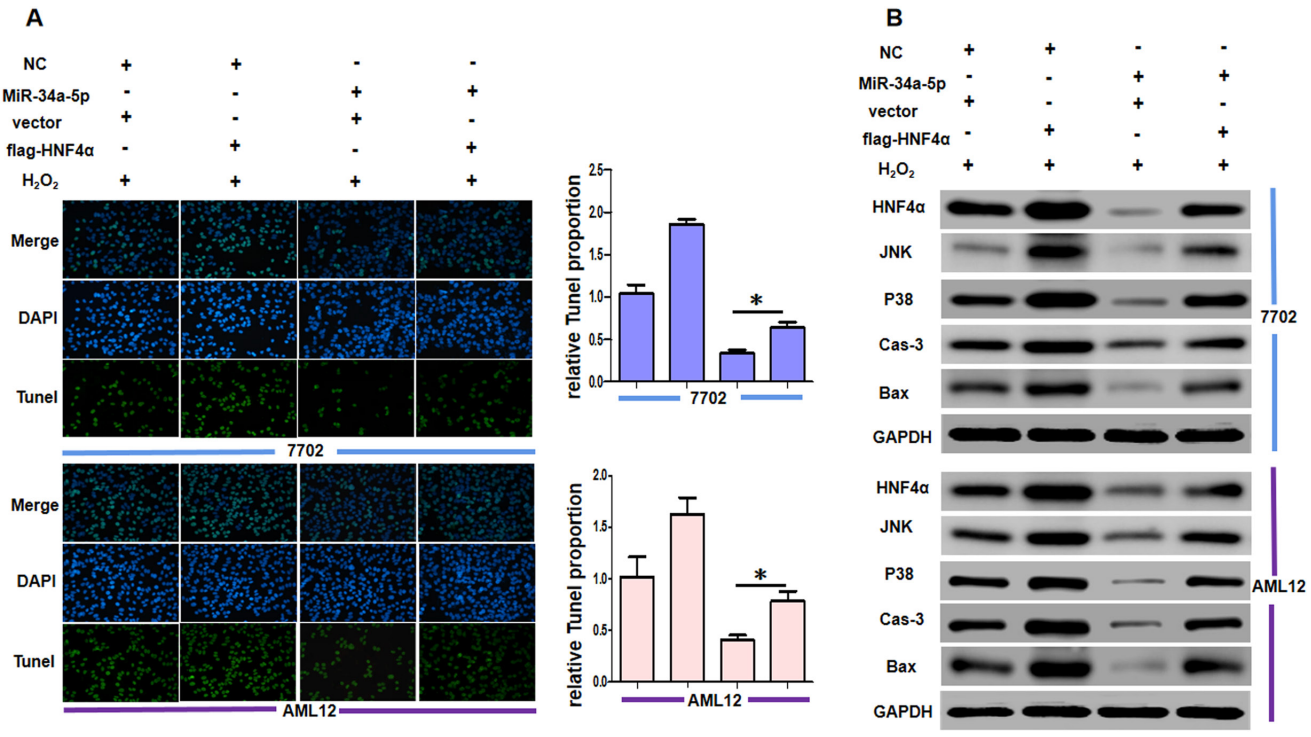

Figure 5. hsa-miR-34a-5p attenuated hepatic I/R injury via targeting HNF4α. (A) TUNEL analysis comparing hepatocellular apoptosis in flag-HNF4α+agomir-NC group, agomir-NC group, flag-HNF4α+agomir-miR-34a-5p group, and agomir-miR-34a-5p group. (B) Western blot analysis comparing HNF4α, JNK, P38, cas-3, and Bax expression in flag-HNF4α+agomir-NC group, agomir-NC group, flag-HNF4α+agomir-miR-34a-5p group, and agomir-miR-34a-5p group (Graphs with error bars in Supplementary Figure 1). All experiments were performed in triplicate, and data were represented as mean ± S.D. *$P < .05$ compared with sham group.

DISCUSSION

The main mechanism of hepatic ischemia–reperfusion injury is the destruction of hepatocyte mitochondria by a large amount of reactive oxygen species during reperfusion. Subsequently, inflammatory factors induce a series of cells such as kupffer cells, CD4 cells, neutrophils, and other cells to infiltrate, and these cells produce interleukins-1, interleukin-6, tumor necrosis factor-a, and chemokines that further enhance the inflammatory response, leading to hepatocyte injury and apoptosis and aggravating liver damage.[17-19] A number of studies have shown that the expression of miR-34a differs in organ ischemia–reperfusion injuries.[20-23] In this study, we first collected blood samples from 60 patients undergoing hepatectomy and found that plasma ALT and AST levels were lower in the high-expression miR-34a-5p group than in the low-expression miR-34a-5p group. To further analyze the role of miR-34a-5p in hepatic I/R injury, we successfully constructed a rat HIRI model and treated it with an intraperitoneal injection of agomir-miR-34a-5p or agomir-NC for 7 days after the surgery. The expression of miR-34a-5p and the pathological changes of the 2 groups were compared. Studies have shown that compared with the HIRI+agomir-NC group, HIRI+gomir-miR-34a-5p group exhibited significantly decreased serum ALT, AST, liver MDA, TOA, OSI concentration, hepatocellular apoptosis, apoptosis-related proteins (cleavage cas-3, Bax, JNK, and P38) and increased miR-34a-5p expression. These results indicate that miR-34a-5p indeed effectively reduced hepatic I/R injury (Figure 6).

The generation of ROS during organ ischemia–reperfusion injury is an important cause of increased damage. Previous study has reported that reducing miR-34a-5p expression can protect small intestinal ischemia–reperfusion injury by reducing ROS production and apoptosis in intestinal endothelial cells through SIRT1 antioxidant pathway.[24] Therefore, in vitro cell injury models generally use H_2O_2 to induce oxidative stress damage to simulate organ I/R injury. To verify the protective effect of miR-34a-5p on hepatic ischemia–reperfusion injury, we used H_2O_2 to treat 7702 and AML12 cells to establish an in

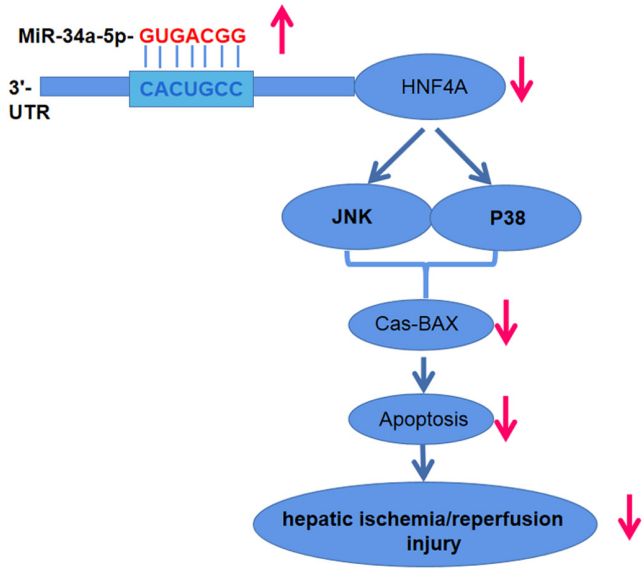

Figure 6. Schematic representation of hsa-miR-34a-5p in regulating hepatic cells apoptosis.

vitro oxidative stress model. The results showed that the apoptosis rate of AML12 and 7702 cells transfected with agomir-miR-34a-5p was significantly lower than that of transfected with agomir-NC, indicating that miR-34a-5p exerted protective effects against H_2O_2-induced oxidative stress. The JNK/P38 signaling pathway is an important pathway for cell regulation and plays an important role in various pathological and physiological processes such as cell cycle, reproduction, apoptosis, and cell stress.[25] Studies have shown that blocking the activation of the JNK/P38 signaling pathway can reduce H_2O_2-induced apoptosis.[26,27] We found that miR-34a-5p downregulated the expression of JNK/P38 in AML12 and 7702 cells. After transfection of agomir-miR-34a-5p, the expression of apoptosis-related protein cleavage cas-3 and Bax was also decreased, indicating that miR-34a -5p reduces H_2O_2-induced apoptosis in AML12 and H_2O_2 cells by inhibiting JNK/P38 signaling pathway.

By searching the PicTar and TargetScan databases, we selected HNF4α as one of the target genes of miR-34a-5P. In vitro cell experiments showed that the expression of HNF4α mRNA and protein in cells with transfected miR-34a-5p was significantly downregulated. By predicting the binding site through the website, we constructed a luciferase reporter plasmid with a mutation binding site and a wild-type binding site, which were transfected into AML12 and 7702 cells with miR-34a-5P and agomir-NC, respectively. Together, these results demonstrate that HNF4α is a downstream target gene of miR-34a-5p. Hepatocyte nuclear factor 4α is an orphan nuclear receptor commonly known as the master regulator of hepatic differentiation, owing to the large number of hepatocyte-specific genes it regulates.[28-29] Some studies have shown that miR-34a can downregulate HNF4α, thereby affecting the function of cells or organs.[30-34] A new study found that HNF4α is a key transcription factor in the repair of renal ischemia–reperfusion injury.[35] Therefore, we hypothesized that miR-34a-5P protects liver I/R injury by acting on HNF4α and we analyzed the activity of HNF4α and JNK/P38 pathway. Our results indicate that miR-34a-5p reduces the expression of apoptosis proteins (cleavage cas-3 and Bax) by downregulating HNF4α, which effectively alleviated cells from oxidative stress damage.

In conclusion, our study showed that miR-34a-5p protected the liver from I/R injury through downregulating HNF4α to inhibit the JNK/P38 signaling pathway. Our findings may help develop new treatments for hepatic I/R injury.

Ethics Committee Approval: The study was approved by the animal management committee of the First Affiliated Hospital of Nanchang University.

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Y.S.; Design – X.Z., Y.S., J.Y.; Supervision – Y.S., G.W., J.Y.; Resources –Y.S., J.Y.; Materials – Y.S., G.W.; Data Collection and/or Processing – X.Z., G.W., N.L., J.Y.; Analysis and/or Interpretation – X.Z., N.L., B.Y.; Literature Search – X.Z., B.Y., J.Y.; Writing Manuscript – X.Z.; Critical Review – Y.S.

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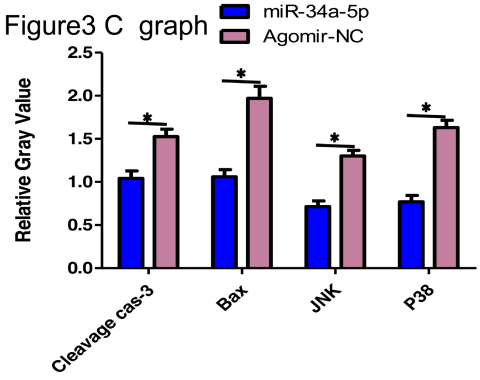
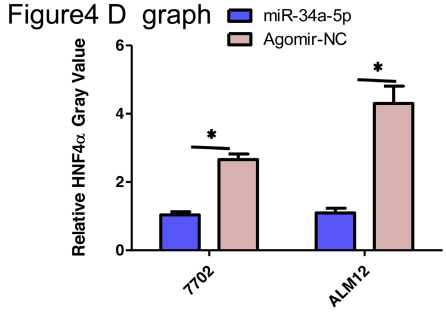
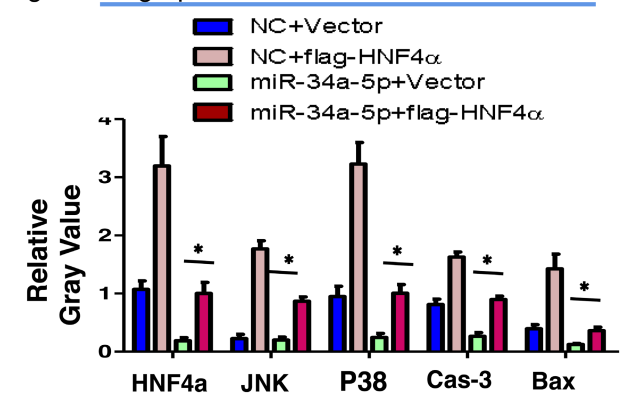
REFERENCES

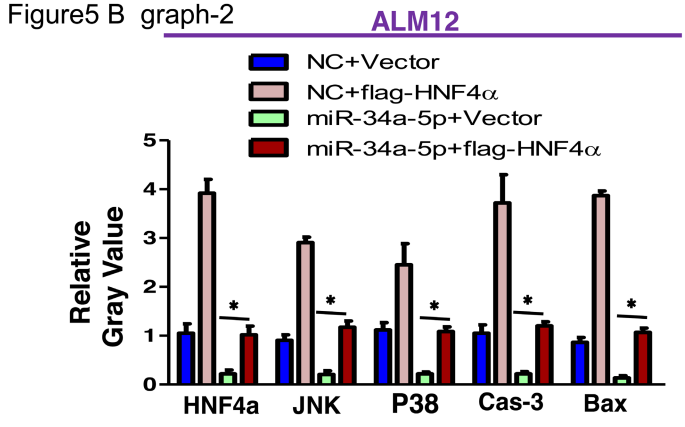
1. Ferrari RS, Andrade CF. Oxidative stress and lung ischemia-reperfusion injury. Oxid Med Cell Longev. 2015;2015:590987. [CrossRef]
2. Weigand K, Brost S, Steinebrunner N, Büchler M, Schemmer P, Müller M. Ischemia/reperfusion injury in liver surgery and transplantation: pathophysiology. HPB Surg. 2012;2012:176723. [CrossRef]
3. Clavien PA, Harvey PR, Strasberg SM. Preservation and reperfusion injuries in liver allografts: an overview and synthesis of current studies. Transplantation. 1992;53(5):957-978. [CrossRef]

4. Ibrahim SG, El-Emam SZ, Mohamed EA, Abd Ellah MF. Dimethyl fumarate and curcumin attenuate hepatic ischemia/reperfusion injury via Nrf2/HO-1 activation and anti-inflammatory properties. Int Immunopharmacol. 2020;80:106131. [CrossRef]
5. Konishi T, Lentsch AB. Hepatic ischemia/reperfusion: mechanisms of tissue injury, repair, and regeneration. Gene Expr. 2017;17(4):277-287. [CrossRef]
6. Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. Hepatology. 2000;32(2):169-173. [CrossRef]
7. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-297. [CrossRef]
8. Novák J, Olejníčková V, Tkáčová N, Santulli G. Mechanistic role of microRNAs in coupling lipid metabolism and atherosclerosis. Adv Exp Med Biol. 2015;887:79-100. [CrossRef]
9. Tétreault N, De Guire V. miRNAs: their discovery, biogenesis and mechanism of action. Clin Biochem. 2013;46(10-11):842-845. [CrossRef]
10. Tahamtan A, Teymoori-Rad M, Nakstad B, Salimi V. Anti-inflammatory microRNAs and their potential for inflammatory diseases treatment. Front Immunol. 2018;9:1377. [CrossRef]
11. Mard SA, Akbari G, Dianat M, Mansouri E. The effect of zinc sulfate on miR-122, miR-34a, antioxidants, biochemical and histopathological parameters following hepatic ischemia/reperfusion injury in rats. Biol Trace Elem Res. 2019;188(2):434-440. [CrossRef]
12. Li T, Gu J, Yang O, Wang J, Wang Y, Kong J. Bone marrow mesenchymal stem cell-derived exosomal miRNA-29c decreases cardiac ischemia/reperfusion injury through inhibition of excessive autophagy via the PTEN/Akt/mTOR signaling pathway. Circ J. 2020;84(8):1304-1311. [CrossRef]
13. Li Y, Luo Y, Li B, Niu L, Liu J, Duan X. miRNA-182/Deptor/mTOR axis regulates autophagy to reduce intestinal ischaemia/reperfusion injury. J Cell Mol Med. 2020;24(14):7873-7883. [CrossRef]
14. Jiang C, Dong N, Feng J, Hao M. MiRNA-190 exerts neuroprotective effects against ischemic stroke through Rho/Rho-kinase pathway. Pflugers Arch. 2021;473(1):121-130. [CrossRef]
15. Qi Y, Zhang K, Li P, Wu Z. Down-regulating miR-217-5p protects cardiomyocytes against ischemia/reperfusion injury by restoring mitochondrial function via targeting SIRT1. Inflammation. 2021;44(1):383-396. [CrossRef]
16. Wang Z, Wang Z, Wang T, Yuan J, Wang X, Zhang Z. Inhibition of miR-34a-5p protected myocardial ischemia reperfusion injury-induced apoptosis and reactive oxygen species accumulation through regulation of notch receptor 1 signaling. Rev Cardiovasc Med. 2019;20(3):187-197. [CrossRef]
17. Klune JR, Tsung A. Molecular biology of liver ischemia/reperfusion injury: established mechanisms and recent advancements. Surg Clin North Am. 2010;90(4):665-677. [CrossRef]
18. Walsh KB, Toledo AH, Rivera-Chavez FA, Lopez-Neblina F, Toledo-Pereyra LH. Inflammatory mediators of liver ischemia-reperfusion injury. Exp Clin Transplant. 2009;7(2):78-93.
19. Arii S, Teramoto K, Kawamura T. Current progress in the understanding of and therapeutic strategies for ischemia and reperfusion injury of the liver. J Hepatobiliary Pancreat Surg. 2003;10(3):189-194. [CrossRef]
20. Huang X, Gao Y, Qin J, Lu S. The role of miR-34a in the hepatoprotective effect of hydrogen sulfide on ischemia/reperfusion injury in young and old rats. PLoS One. 2014;9(11):e113305. [CrossRef]
21. Li W, Jin S, Hao J, Shi Y, Li W, Jiang L. Metformin attenuates ischemia/reperfusion-induced apoptosis of cardiac cells by down-regulation of p53/microRNA-34a via activation of SIRT1. Can J Physiol Pharmacol. 2021;99(9):875-884. [CrossRef]
22. Huang X, Gao Y, Qin J, Lu S. The mechanism of long non-coding RNA MEG3 for hepatic ischemia-reperfusion: mediated by miR-34a/Nrf2 signaling pathway. J Cell Biochem. 2018;119(1):1163-1172. [CrossRef]
23. Wang X, Yuan B, Cheng B, et al. Crocin alleviates myocardial ischemia/reperfusion-induced endoplasmic reticulum stress via regulation of miR-34a/Sirt1/Nrf2 pathway. Shock. 2019;51(1):123-130. [CrossRef]
24. Wang G, Yao J, Li Z, et al. miR-34a-5p inhibition alleviates intestinal ischemia/reperfusion-induced reactive oxygen species accumulation and apoptosis via activation of SIRT1 signaling. Antioxid Redox Signal. 2016;24(17):961-973. [CrossRef]
25. Cederbaum AI, Lu Y, Wang X, Wu D. Synergistic toxic interactions between CYP2E1, LPS/TNFα, and JNK/p38 MAP kinase and their implications in alcohol-induced liver injury. Adv Exp Med Biol. 2015;815:145-172. [CrossRef]
26. Tang C, Liang J, Qian J, et al. Opposing role of JNK-p38 kinase and ERK1/2 in hydrogen peroxide-induced oxidative damage of human trophoblast-like JEG-3 cells. Int J Clin Exp Pathol. 2014;7(3):959-968.
27. Xue H, Yan K, Zhao X, et al. Pretreatment with pPolyHb attenuates H2O2-induced endothelial cell injury through inhibition of JNK/p38 MAPK pathway by upregulation of heme oxygenase-1. Artif Cells Nanomed Biotechnol. 2015;43(3):163-173. [CrossRef]
28. Lau HH, Ng NHJ, Loo LSW, Jasmen JB, Teo AKK. The molecular functions of hepatocyte nuclear factors - In and beyond the liver. J Hepatol. 2018;68(5):1033-1048. [CrossRef]
29. Babeu JP, Boudreau F. Hepatocyte nuclear factor 4-alpha involvement in liver and intestinal inflammatory networks. World J Gastroenterol. 2014;20(1):22-30. [CrossRef]
30. Wang Z, Burke PA. The role of microRNAs in hepatocyte nuclear factor-4alpha expression and transactivation. Biochim Biophys Acta. 2013;1829(5):436-442. [CrossRef]
31. Xu Y, Zalzala M, Xu J, Li Y, Yin L, Zhang Y. A metabolic stress-inducible miR-34a-HNF4alpha pathway regulates lipid and lipoprotein metabolism. Nat Commun. 2015;6:7466. [CrossRef]
32. Salloum-Asfar S, Arroyo AB, Teruel-Montoya R, et al. MiRNA-based regulation of hemostatic factors through hepatic nuclear Factor-4 alpha. PLoS One. 2016;11(5):e0154751. [CrossRef]
33. Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T. MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. J Biol Chem. 2010;285(7):4415-4422. [CrossRef]
34. Li Z, Chen H. miR-34a inhibits proliferation, migration and invasion of paediatric neuroblastoma cells via targeting HNF4α. Artif Cells Nanomed Biotechnol. 2019;47(1):3072-3078. [CrossRef]
35. Wilflingseder J, Willi M, Lee HK, et al. Enhancer and super-enhancer dynamics in repair after ischemic acute kidney injury. Nat Commun. 2020;11(1):3383. [CrossRef]









Supplementary Figure 1