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**miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury *via* decreasing the Atg12-Atg5 conjugate**

Shipeng Li *et al*. miR-30b inhibits autophagy *via* decreasing Atg12-Atg5

Shi-Peng Li, Jin-Dan He, Zhen Wang, Yao Yu, Hai-Ming Zhang, Jian-Jun Zhang, Zhong-Yang Shen

**Shi-Peng Li, Jin-Dan He, Zhen Wang, Yao Yu, Hai-Ming Zhang, Jian-Jun Zhang, Zhong-Yang Shen,** First Central Clinical College of Tianjin Medical University, Tianjin 300192, China

**Hai-Ming Zhang, Jian-Jun Zhang, Zhong-Yang Shen,** Oriental Organ Transplant Center of Tianjin First Central Hospital, Tianjin 300192, China

**Shi-Peng Li, Jin-Dan He, Zhen Wang, Yao Yu, Hai-Ming Zhang, Jian-Jun Zhang, Zhong-Yang Shen,** Key Laboratory of Organ Transplantation of Tianjin, Tianjin 300192, China

**Author Contributions:** Li SP, Wang Z and Yu Y performed the majority of experiments and analyzed the data; He JD, Wang Z and Yu Y performed the molecular investigations; He JD, Wang Z and Yu Y participated equally in treatment of animals; Zhang HM, Zhang JJ and Shen ZY designed and coordinated the research; Li SP and Zhang JJ wrote the paper; LiSP, HeJD, Wang Z and Yu T contributed equally to this work.

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**Correspondence to: Jian-Jun Zhang, MD, PhD,** First Central Clinical College of Tianjin Medical University, Oriental Organ Transplant Center of Tianjin First Central Hospital, Key Laboratory of Organ Transplantation of Tianjin, No. 24 Fukang Road, Nankai District, Tianjin 300192, China. zhangjianjun9999@yeah.net

**Telephone:** +86-22-23626600

**Fax:** + 86-22-23626600

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**Abstract**

**AIM:** To explore the role and potential mechanism of miR-30b regulating autophagy in hepatic IRI.

**METHODS:** A hepatic IRI model was performed in C57BL/6 mice, and AML12 cells were immersed in mineral oil for 1 h, and then cultured in complete DMEM/F12 to simulate IRI model. Mice and Cells were transfected with miR-30b agomir/mimics or antagomir/inhibitor to examine the effect of miR-30b on regulating autophagy to promote hepatic IRI. The expression of miR-30b was measured by real-time PCR. Apoptotic cells were detected by TUNEL staining, and cell viability was detected by MTT. The expression of LC3, Atg12, Atg5, P62 and Caspase-3 were detected by the western blotting analysis.

**RESULTS:** miR-30b levels were significantly down-regulated after hepatic IR, and the numbers of autophagosomes increased in response to IR, demonstrating that low levels of miR-30b could promote hepatic IRI as revealed by reductions in vivo and vitro. Furthermore, we found that miR-30b interacted with Atg12-Atg5 conjugate by binding to Atg12. Over-expression of miR-30b diminished Atg12 and Atg12-Atg5 conjugate levels which promoted autophagy in response to IR; However, low level-expressions of miR-30b were associated with increased Atg12-Atg5 conjugate levels and increased autophagy.

**CONCLUSION:** miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury via decreasing the Atg12-Atg5 conjugate.

**Key words:** miR-30b; Autophagy; Atg12-Atg5 conjugate; Hepatic ischemia-reperfusion injury

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**Core tip:** miR-30b levels were significantly down-regulated after hepatic ischemia-reperfusion injury, and the numbers of autophagosomes increased in response to ischemia-reperfusion, demonstrating that low levels of miR-30b could promote hepatic ischemia-reperfusion injury as revealed by reductions in cells viability in vitro. Over-expression of miR-30b diminished Atg12 and Atg12-Atg5 conjugate levels which promoted autophagy in response to hepatic ischemia-reperfusion injury. Therefore, miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury via decreasing the Atg12-Atg5 conjugate.

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**INTRODUCTION**

Hepatic IRI represents an important factor with regard to the prognosis of surgical outcomes and patient survival, as well as the protection of hepatic cells [1]. A key consideration regarding liver function has been revealed from recent data which have demonstrated that autophagy represents a principal component of hepatology[2]. Autophagy consists of a tightly regulated intracellular catabolic pathway involving lysosomal degradation of cytoplasmic organelles and proteins[3].miRNAs are closely linked to virtually all fundamental biological pathways[4]. Importantly, miRNAs play critical roles in a broad range of biological processes including proliferation, differentiation, apoptosis and stress responses[5].

Recent findings have indicated some novel roles for miRNAs in the regulation of autophagy[6,7]. In this report, we focussed on miRNAs with direct autophagic implications, as exerted either through putative core components of autophagy machinery or through less well-characterized mechanisms. In specific, the 3′-UTR of the autophagy associated gene 12 (Atg12) contains the predicated target sites for miRNA-30b (miR-30b), which have been identified by luciferase reporter gene assays. The possibility exists that miR-30b might contribute to alleviating IRI via modulating autophagy through targeting Atg12. In this study, we attempted to determine whether miR-30b modulates autophagy and thus alleviate hepatic IRI. Specifically, we over- or down-regulated expression of miR-30b to examine the effects of miR-30b on Atg12 and Atg12-Atg5 conjugate levels regulating autophagy in hepatic IRI. Our data indicate that miR-30b might serve as a novel therapeutic target regulating autophagy in hepatic IRI.

**MATERIALS AND METHODS**

***Animals and cell line***

Male C57BL/6 mice (7-8 wk old, 23 ± 3 g), purchased from the experimental animal center of the PLA Military Medical Science Academy. All animals received humane care according to established standards and were maintained in an air-conditioned animal room at 25 ℃ with free access to water and food. All protocols conformed to the National Institute of Health (NIH) guidelines and all animals received care in compliance with the principles of laboratory animal care. The AML12 cell line (mouse hepatic cell) was purchased from the American Type Culture Collection (ATCC, VA, United States). The study was performed according to Tianjin Medical University Institutional review board guidelines and the protocol was approved by the Institutional review board.

***Reagents and antibodies***

DMEM/F12 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY); miR-30b-5p mimics/agomir, miR-30b-5p inhibitor/antagomir, miR-NC, Atg12 siRNA and RiboFECTTM CP Reagent were purchased from RiboBio Co., Ltd. (Guangzhou, China); Rapamycin and 3-MA were purchased from SelleckInc (United States). The In Situ Cell Death Detection Kit, TMR red and SYBR Green qRT-PCR Master Mix were purchased from Roche Diagnostics GmbH (Mannheim, Germany); Trizol and Lipofectamine 2000 were obtained from Invitrogen (Rockville, MD). Antibody Atg12, LC3, P62, Caspease-3, Cleave Caspease-3, PARP1, β-actin, and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology Inc (United States).

***Animal model and treatment***

Thesegmental (70%) hepatic ischemia model was performed according to that described previously[1], and there were 6 mice in sham group while the 24 mice in the IR group were divided by reperfusion times consisting of 2, 6, 12, and 24 h. The miR-30b-5p agomir group (*n* = 6), miR-30b-5p antagomir group (*n* = 6) and miR-NC group (*n* = 6). The miR-30b-5p agomir (10 nmol/L), antagomir (10 nmol/L) or miR-NC (10 nmol/L) was administered tail intravenous injection 24 h prior to ischemia.

***Serology detection***

Serum AST and ALT levels of the mice in all groups were determined with use of a commercial assay kit (Nanjing Jiancheng Biological Technology, China). Enzyme activities were expressed as international units per liter (U/L).

***Histology and transmission electron microscopy***

Samples of liver were fixed in 4% mediosilicic isotonic formaldehyde for 24 h, dehydrated and embedded in paraffin. Five micrometer-thick sections were cut from each paraffinembedded tissue and stained with H&E to evaluate the degree of liver damage. In addition, liver and cell samples were placed in 1% glutaraldehyde and post-fixed with 2% osmium tetroxide. The cell pellets or sections were embedded in epon resin. The data were quantified by counting the number of autophagosomes per cross-sectioned cell.

***Cell culture and treatment***

AML12 cells were plated at a density of 2×105 cells/mL in 6-well plates and divided into five groups[8]: (1) control group Cells were cultured in DMEM/F12 without treatment; (2) IR group Cells were immersed in mineral oil (1ml/well) for 1 h to simulate ischemia, then cultured in DMEM/F12 for 12 h to simulate reperfusion; (3) miR-30b-5p mimics group Cells were transfected with 50 nmol/L miR-30b-5p mimics or miR-NC using RiboFECTTM CP Reagent for 24 h according to the manufacturer’s protocol, followed by reperfusion as described above; (4) miR-30b-5p inhibitor group Cells were transfected with 50 nmol/L miR-30b-5p inhibitor or miR-NC, followed by reperfusion; and (5) Atg12 siRNA group Cells were treated with Atg12 siRNA or siRNA-NC for 24 h, followed by reperfusion.

***MTT bioassay***

Cells were seeded onto 96-well plates (5 × 104 cells/well) and after culture for 24h at 37℃, subjected to reperfusion as described above. Fresh medium was then added to each well together with 20 μL MTT solution (5 mg/mL), and the plate was incubated at 37 ℃ for 4 h. The medium was then removed and 200 μL DMSO was added per well. The optical density (OD) of each well was determined with a test wavelength of 490 nm.

***Confocal fluorescent microscopic detecting autophagy***

AML12 cells were cultured in 6-well plates to 60%-70% confluence. The cells were transfected with tandem GFP-RFP-LC3 adenovirus (Hanbio, Shanghai, China) immediately according to GFP-RFP-LC3 instruction manual to further confirm autophagy induction.

***Immunocytochemistry***

The streptavidin-peroxidase (SP) staining technique was used to detect protein following antigen retrieval by microwave treatment. After blocking endogenous peroxidase activity by incubating in 3% H2O2 for 15 min, specimens were incubated with antibodies (PCNA and Caspase-3) at 4 °C overnight. Specimens were incubated at room temperature for 1 h with the secondary antibody, and then diaminobenzidine (DAB) solution was used. Counterstaining was performed with hematoxylin.

***QRT-PCR***

Total RNA was isolated by Trizol and 1μg of RNA used for reverse transcription was prepared as described above. Real-time PCR was performed in a total volume of 25 μL reaction mixture. U6 orβ-actin was used as an internal control and the expression levels was calculated using MxPro software (Version 4.0, Stratagene).

***Luciferase reporter gene assays***

The miRWalk database was used to predict the binding site on the 3´-UTR of miR-30b, and this database combines several bioinformatic platforms including TargetScan 4.2, miRBase, and miRanda. Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions[9-11]. Cells were transferred into 24-well plates at 3 × 104 cells per well. After 24 h, the cells were transiently co-transfected with pRL-TK plasmid (Promega), and various constructs containing different lengths of the Atg12 5’-flanking region or pGL3-Basic. The luciferase activities were measured as the manufacturer’s instructions.

***Western blot analysis***

Protein samples were harvested from the mice livers and AML12 cells as obtained from the different groups. The proteins were then transferred from the gel to the nitrocellulose membranes. Probed with the antibodies Atg12, LC3, P62, Caspease-3, Cleave Caspease-3, RARP1 and β-actin. Bound antibodies were then visualized using an ECL detection kit with an appropriate HRP-conjugated secondary antibody.

***Apoptosis analysis using TUNEL***

TUNEL reactions were performed using an In Situ Cell Death Detection Kit, TMR red. For quantification, the mean number of TUNEL-positive cells, as determined using 200× in five different fields were averaged.

***Statistical analysis***

All data are presented as means±SD. Differences among groups were analyzed using a one-way analysis of variance followed by the Student-Newman-Keuls post-hoc test. The SPSS software 19.0 was used for these analyses and a *P* < 0.05 was required for results to be onsidered statistically significant.

**RESULTS**

***Hepatic IRI alters miR-30b, Atg12 and Atg5 mRNA expression in mice livers***

With IR mouse model, miR-30b-5p expression levels gradually decreased (*P* < 0.05) after reperfusion, however, levels of Atg12 and Atg5 mRNA increased thereafter (*P* < 0.05) as compared with the Sham group (Figure 1A).

***Alterations of autophagy in mice livers induced by ischemia reperfusion injury***

As shown in Figure 1B, the expression of Atg12, Atg12-Atg5 conjugate and LC3II was up-regulated as a function of time following reperfusion (*P* < 0.05). Meanwhile, Cleave Caspase-3 expression showed a temporally dependent increase as a function of time following reperfusion.

***Alterations of liver pathological changes and serum AST, ALT levels***

Pathological analyses as presented in Figure 1C revealed considerable hepatocyte edema, congestion and apoptosis was observed at 6-24 h post-reperfusion as compared with the Sham group. The changes in serum AST and ALT levels of mice as a function of the different reperfusion time points is illustrated in Figure 1D. Serum AST and ALT levels gradually increased (*P* < 0.05), reaching peaked at 12 h (*P* < 0.001) following reperfusion. Next, we evaluated autophagic vacuoles using transmission electron microscopy (TEM). Autophagosomes, containing partially degraded cytoplasmic material, were clearly observed with TEM visualization (Figure 1E). The basal number of autophagosomes within the IR group was increased relative to the Sham group (*P* < 0.001).

***miR-30b can alleviate mouse hepatic ischemia-reperfusion injury***

To clarify whether miR-30b can alleviate HIRI, we over- or down-regulated expression of miR-30b in mice after tail intravenous injection of miR-30b-5p agomir or antagomir at 12 h following reperfusion. As was shown in Figure 2A, we found that miR-30b-5p agomir significantly decreases the histopathologic changes of livers induced by IR treatment, but miR-30b-5p antagomir can aggratate the changes. Compared with miR-NC group, serum AST and ALT levels in the miR-30b-5p agomir group mice decreased while that of the miR-30b-5p antagomir group mice increased as a function of time following reperfusion (*P* < 0.05; Figure 2B). As illustrated in Figure 2C, the number of TUNEL-positive cells were significantly decreased in compared with that of the miR-NC group (*P* < 0.05), however, miR-30b-5p antagomir increased the number of TUNEL-positive cells compared with that of the miR-NC group (*P* < 0.01). When compared with miR-NC group at the 12 h reperfusion time, the miR-30b-5p agomir resulted in a significant increase in PCNA expression but decrease in Caspase-3, Cleave Caspase-3 and PARP1 expression. In contrast, the miR-30b-5p antagomir could decrease in PCNA expression but increase in Caspase-3, Cleave Caspase-3 and PARP1 expression (Figure 2D and E). These findings demonstrate that miR-30b can alleviate hepatic ischemia-reperfusion injury.

***miR-30b inhibits autophagy by sequestering Atg12***

Based upon information contained within the bio-informatics database, we hypothesized that the miR-30b binding site was at the 3´-UTR of Atg12, and a luciferase reporter assay was performed to determine the effects of miR-30b on the Atg12 mRNA 3′-UTR (Figure 3A). When examined at 12 h post-reperfusion, an over expression of miR-30b significantly reduced Atg12 mRNA and protein levels within AML12 cells transfected with miR-30b-5p mimics as compared with that observed within the miR-NC (*P* < 0.05). Moreover, levels of Atg12-Atg5 conjugate and LC3II decreased after transfection of miR-30b-5p mimics when compared with that of the miR-NC (*P* < 0.05). In contrast, the results in AML12 cells treated with miR-30b-5p inhibitor were opposite. Our findings indicate that miR-30b could decrease Atg12 and Atg12-Atg5 conjugate in AML12 cells.

We performed an Ad-GFP-RFP-LC3 to examin the potential role of miR-30b in autophagy after AML12 cells were subjected to IR. The appearance of GFP- or RFP-LC3 dots within the cytoplasm reflects the recruitment of LC3 proteins to autophagosomes. When autophagy inducts, both GFP and RFP are expressed as yellow dots representing autophagosomes after the images emerged. When autophagosomes fuse with lysosomes and form autolysosomes, the GFP degrades in an acid environment, but RFP-LC3 maintains showing as red dots. Confocal immunofluorescence experiment was performed to ensure the increase of LC3. The Ad-GFP-RFP-LC3 was transfected into AML12 cells to confirm the induction of autophagy. As the results showed in Figure 3D, miR-30b could inhibit the induction of autophagosomes significantly. We observed both fluorescent proteins showed successfully after the infection with the Ad-GFP-RFP-LC3. There were significant decreases of yellow dots with marginal elevations in red dots in the miR-30b-5p mimics group than in miR-NC or miR-30b-5p inhibitor group (*P* < 0.001). Next, we evaluated autophagic vacuoles using TEM. Autophagosomes were clearly observed with TEM visualization (Figure 3E). The basal number of autophagosomes within the miR-30b-5p mimics group was decreased relative to the miR-30b-5p inhibitor or miR-NC group (*P* < 0.001). Taken together, the results show that miR-30b inhibits autophagic flux by sequestering Atg12.

***miR-30b inhibits autophagy to alleviate AML12 cell ischemia-reperfusion injury by targeting Atg12***

In order to assess whether cells become more resistant to IRI dependent on autophagy in HIRI model, we examined the effects of either activating or inhibiting autophagy with use of the rapamycin and 3-MA. Rapamycin decreased the survival ratio of AML12 cells induced by IR treatment, but 3-MA inhibited the changes (*P* < 0.05). Moreover, Rapamycin increased the levels of LC3Ⅱ and decreased P62 expression, however, 3-MA decreased the levels of LC3Ⅱ and increased P62 expression. These data identified that activating autophagy could aggratate HIRI. miR-30b-5p mimics increased viability of AML12 cells treated responding to IR (*P* < 0.05), but viability of AML12 cells treated with miR-30b-5p inhibitor decreased responding to IR (*P* < 0.05). As demonstrated in Figure 4D, Atg12 siRNAs significantly down-regulated Atg12 expression in AML12 cells. The viability of AML12 cells treated with miR-30b-5p mimics or inhibitor responding to IR (Figure 4E) was enhanced by siRNA knockdown of Atg12. At the same time, AML12 cells were also treated with miR-30b-5p mimics/inhibitor or Atg12 siRNA to investigate potential interactions between the miR-30b and Atg12-Atg5 conjugate during IR. At 12 h post-reperfusion, siRNA-mediated knockdown of Atg12 contributed to Atg12-Atg5 conjugate and LC3II protein levels inhibited by miR-30b-5p mimics, while Atg12 siRNA could decrease the levels of Atg12-Atg5 conjugate and LC3II induced by miR-30b-5p inhibitor (Figure 4F). These data suggest that miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury by targeting Atg12.

**DISCUSSION**

Autophagy plays a pivotal role in cellular homeostasis and adaptation to adverse environments[12,13], although the regulation of this process remains incompletely understood[14], where it provides a cytoprotective role resulting in cellular adaptation and survival[15]. Autophagy is regarded as a natural and essential defense mechanism against inflammatory, damnification, and oncotherapy[16]. Hence regulation of autophagy pathway has been implicated in the pathogenesis of numerous human diseases. A number of studies on autophagy and liver diseases have rapidly focussed on liver ischemia reperfusion[17-20]. miR-30b, a member of the miR-30 family, has been suggested to play a role in the differentiation of several cell types[21]. The miR-30 family is also involved in the control of structural changes in the extracellular matrix of the myocardium[22] and in the regulation of the apoptosis[23].

In this study, we discovered that miR-30b down-regulated in mice livers subjected to IR. In addition, the expression of Atg12 and LC3II up-regulated, and the numbers of autophagosomes observed in the IR group increased as a function of time following reperfusion. miR-30b expression decreases in response to hepatic IRI and is accompanied by a corresponding activation of autophagy. The induction of autophagy represents an initial response to ischemia-reperfusion in mice livers, while Atg12 and Atg12-Atg5 conjugate expression decreased as a function of time following reperfusion. To clarify whether miR-30b can alleviate HIRI, we over- or down-regulated expression of miR-30b in mice after tail intravenous injection of miR-30b-5p agomir or antagomir. miR-30b-5p agomir significantly decreases the histopathologic changes of livers induced by IR treatment, and the number of TUNEL-positive cells were significantly decreased. However, down-regulated expression of miR-30b could aggratate the histopathologic changes. Proliferating cell nuclear antigen (PCNA), a subunit of the mammalian DNA polymerase delta, is synthesized primarily during the S phase of the cell cycle[24]. PCNA is a relay molecule that functions as a molecular integrator for proteins involved in the control of the cell cycle, DNA repair and cell death[25]. Therefore, PCNA is a convincing marker to distinguish proliferating cells. The miR-30b-5p agomir resulted in a significant increase in PCNA expression but decrease in Caspase-3, Cleave Caspase-3 and PARP1 expression. In contrast, down-regulated expression of miR-30b could decrease in PCNA expression but increase in Caspase-3, Cleave Caspase-3 and PARP1 expression. These findings demonstrate that miR-30b can alleviate hepatic ischemia-reperfusion injury.

Autophagosome formation requires two ubiquitin-like conjugation systems, the Atg12 and LC3 systems[26]. The Atg12 system is located upstream of the LC3 system in the context of Atg protein organization. Atg12 is finally conjugated to Atg5, forming the irreversible Atg12-Atg5 complex, which strongly enhances the formation of LC3-phosphatidylethanolamine conjugation[27]. Cav-1 also regulated ATG12-ATG5 conjugate during autophagosome formation, and Cav-1 competitively interacts with the ATG12-ATG5 system to suppress the formation and function of the latter in lung epithelial cells[28]. Based upon miRNA target gene prediction, we identified the 3´-UTR area of the Atg12 gene as being matched with miR-30b.

Our data shown that up-regulated expression of miR-30b significantly increased cell viability as a function of time following reperfusion, while the miR-30b inhibitor significantly decreased cells viability. Related to these findings, a significant decrease of LC3 dots in AML12 transfected with miR-30b-5p mimics as compared with miR-NC group. The number of LC3 dots increased in the miR-30b inhibitor group relative to the miR-30b mimics group. These results indicate that miR-30b increased the viability of hepatocyte induced by IR via inhibiting autophagy. Over-expression of miR-30b significantly reduced Atg12 and Atg12-Atg5 conjugate protein levels after AML12 cells transfected with miR-30b mimics; and LC3II expressions decreased in these cells. The expression of Atg12 and Atg12-Atg5 conjugate protein levels increased after AML12 cells were transfected with the miR-30b inhibitor, while LC3II began to up-regulate at the same time. Collating these findings it seems clear that miR-30b can decrease Atg12 and Atg12-Atg5 conjugate expression thereby down-regulating autophagy to alleviate hepatic IRI.

Autophagy is a self-digesting process that occurs in response to stress and plays important roles in the pathogenesis of a variety of diseases [15]. Autophagy functions mainly in a pro-survival capacity for cells to cope with nutrient starvation and anoxia[29,30], however, excessive levels of autophagy within impaired cells can contrarily induce cell death[31]. Oxidative stress may lead to autophagy which induces cell death in cisplatin-induced AKI[32]. In our study, we found that activation of autophagy aggravated HIRI, an effect which was dependent on Atg12 and Atg12-Atg5 conjugate. We also treated AML12 cells with the miR-30b mimics, inhibitor or Atg12 siRNA to investigate whether any potential interaction may exist between miR-30b and Atg12 during IR. Our findings suggest that miR-30b mediated apoptosis to alleviate hepatic IRI, an effect which is dependent on Atg12 activity. As was shown in Figure 5, we found that miR-30b inhibited autophagy to alleviate HIRI via decreasing the Atg12-Atg5 conjugate. This finding may serve as a guide to prevent hepatic ischemia reperfusion injury and provide a future strategy in research areas of ischemia reperfusion injury.

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**COMMENTS**

***Background***

Hepatic ischemia reperfusion injury (HIRI) represents an important clinical problem as related to liver resection or transplantation. miRNAs participate in various hepatic pathophysiological processes via regulating autophagy. miR-30b, a member of the miR-30 family, has been involved in the control of structural changes in in the regulation of the apoptosis. however, the importance and function of miR-30b have not yet been investigated, and whether miR-30b regulating autophagy to alleviate HIRI has not yet been elucidated.

***Research frontiers***

It was reported that miR-30b overexpression had anti-apoptotic effect on early phase of rat myocardial ischemia injury model through targeting KRAS and activating the Ras/Akt pathway, and E2F1-regulated miR-30b suppressed Cyclophilin D to protect heart from ischemia-reperfusion injury and necrotic cell death.

***Innovations and breakthroughs***

This is the first study investigating the roles of miR-30b in inhibiting autophagy to alleviate hepatic ischemia reperfusion injury, and the mechanism is via decreasing the levels of Atg12-Atg5 conjugate. This study may provide a future research strategy in hepatic ischemia reperfusion injury.

***Applications***

This study provides insight into the roles of miR-30b in inhibiting autophagy to alleviate hepatic ischemia reperfusion injury via decreasing the Atg12-Atg5 conjugate. Understanding miR-30b regulating autophagy is involved in process of hepatic ischemia reperfusion injury may facilitate the design of new therapeutic approaches to prevent and cure hepatic ischemia reperfusion injury.

***Terminology***

Atg12 ubiquitin-like conjugation systems is required in autophagosome formation, and Atg12 system is located upstream of the LC3 system in the context of Atg protein organization. Atg12 is finally conjugated to Atg5, forming the irreversible Atg12-Atg5 complex, which strongly enhances the formation of LC3-phosphatidylethanolamine conjugation.

***Peer-review***

This is an intereting paper, and it brings us very important information about the expression of miR-30b and autophagy in the process of hepatic ischemia reperfusion injury. This study found that the miR-30b inhibited autophagy to alleviate HIRI via decreasing the Atg12-Atg5 conjugate. The result is very important for advanced research of hepatic ischemia reperfusion injury.

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**C:\Users\lenovo\Desktop\课题实验\miR-30b-5p\World Journal of Gastroenterology\Fig 1.tif**

**Figure 1 Alterations of miR-30b and autophagy in mouse livers in response to ischemia-reperfusion injury.** A: The expression of miR-30b in mouse livers subjected to IR as determined by qRT-PCR analysis; B: Western blotting for Atg12, LC3 and Cleave Caspase-3 in mice livers; C: IR treatment increases the histopathologic changes in livers (magnification × 200); D: IR treatment increased serum Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) levels in mice compared with Sham; E: TEM images of mouse hepatocytes after ischemia followed by reperfusion at 12 h. Scale bars = 2.0 μm. The data were quantified by counting the number of autophagosomes per cross-sectioned cell. a*P* < 0.05, b*P* < 0.01, c*P* < 0.001 *vs* Sham group. Every experiment was repeated three times.

**C:\Users\lenovo\Desktop\课题实验\miR-30b-5p\World Journal of Gastroenterology\Fig 2.tif**

**Figure 2 miR-30b can alleviate mouse hepatic ischemia-reperfusion injury.** A: HE stain was used to observe histopathologic changes in mice livers (magnification × 200) after tail intravenous injection of miR-30b-5p agomir or antagomir at 12 h following reperfusion; and B: serum AST and ALT levels; C: Cell apoptosis as measured by TUNEL. Representative sections as determined at 12 h post-reperfuion (magnification × 200); D: Immunohistochemisty revealed expression of PCNA and Caspase-3 (magnification × 200); E: Western blot was used to detect expression of Bcl-2, Cleave Caspase-3 and PARP1 in livers, a*P* < 0.05, b*P* < 0.01 *vs* miR-NC group. Every experiment was repeated three times.

**C:\Users\lenovo\Desktop\课题实验\miR-30b-5p\World Journal of Gastroenterology\Fig 3.tif**

**Figure 3 miR-30b inhibits autophagy by sequestering Atg12 in AML12 cells.** A and B: The predicted miR-30b binding site on the Atg12 mRNA 3′-UTR was shown and a luciferase reporter assay was performed to determine the effects of miR-30b on the Atg12 mRNA 3′-UTR; C: Western blot was used to detect expression of Atg12 and LC3 in AML12 cells treated with miR-30b mimics or inhibitor; D: Confocal immunofluorescence of AML12 cells demonstrated increased numbers of GFP-RFP-LC3 dots in the IR group; When autophagy inducts, both GFP and RFP are expressed as yellow dots (autophagosomes). Red dots represent autolysosomes as the GFP degrades in an acid environment. Scale bars = 20 μm; E: TEM images of AML12 cells after ischemia followed by reperfusion at 12 h. TEM images show representative examples of autophagosomes (red arrows). Scale bars = 2.0 μm. And the data were quantified by counting the number of autophagosomes per cross-sectioned cell; a*P* < 0.05, b*P* < 0.01, c*P* < 0.001 *vs* miR-NC group. Every experiment was repeated three times.

**C:\Users\lenovo\Desktop\课题实验\miR-30b-5p\World Journal of Gastroenterology\Fig 4.tif**

**Figure 4 miR-30b alleviate AML12 cell ischemia-reperfusion injury by targeting Atg12 in vitro.** A: The survival ratio of AML12 cells was measured after treated with Rapamycin or 3-MA; B: Western blot was used to detect expression of LC3 and P62 in AML12 cells treated with Rapamycin or 3-MA; C: The survival ratio of AML12 cells was measured after treated with miR-30b mimics or inhibitor; D: AML12 cells were transfected with Atg12 siRNAs, and the Atg12 mRNA and protein level of the target was evaluated using qRT-PCR or western blot analysis. E: The survival ratio of AML12 cells was measured at 12 h post-reperfusion in the absence or presence of miR-30b mimics, the miR-30b inhibitor, or Atg12 siRNA; F: Protein levels of Atg12 and LC3 were analyzed using western blot in AML12 cells at 12 h post-reperfusion in the absence or presence of miR-30b mimics, the miR-30b inhibitor, or Atg12 siRNA; a*P* < 0.05, b*P* < 0.01, c*P* < 0.001 *vs* Control/miR-NC group. Every experiment was repeated three times.

C:\Users\lenovo\Desktop\课题实验\miR-30b-5p\World Journal of Gastroenterology\Fig 5.tif

**Figure 5 Model of miR-30b inhibiting autophagy to alleviate HIRI via decreasing the Atg12-Atg5 conjugate.** The expression of miR-30b, binding site was at the 3´-UTR of Atg12, was significantly down-regulated after hepatic ischemia-reperfusion injury, then levels of Atg12 and Atg12-Atg5 conjugate increased and promoted autophagy, leading to apoptotic cell death. This shows that miR-30b can inhibit autophagy to alleviate hepatic ischemia-reperfusion injury via decreasing the Atg12-Atg5 conjugate.