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MiR-640 inhibition alleviates acute liver injury *via* regulating WNT signaling pathway and LRP1

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Abstract. – OBJECTIVE: Acute liver injury (ALI) is associated with the Kupffer cells (KCs) inflammation and hepatocytes apoptosis. Previous studies have shown that miR-640 is a valid regulator of the Low-density lipoprotein receptor-related protein 1 (LRP 1) which expressed much lower in an inflammatory condition. However, it is unclear whether MiR-640 inhibition protects against ALI by the up-regulation of LRP 1. To explore the regulated mechanism of miR-640 on acute liver injury.

MATERIALS AND METHODS: We analyzed the expression of miR-640 in different times of acute injured liver tissues. Lipopolysaccharide (LPS) was employed in provoking the KCs inflammation to injure liver. We used miR-640 mimic or inhibitor to improve or resist the function of miR-640 to explore miR-640 function to ALI via the target of LRP1.

RESULTS: We showed that the expression of miR-640 markedly increased in LPS-induced acute injured liver tissues. LPS promoted the progress of ALI, and the inhibition of miR-640 could reverse the injured effects of LPS. Moreover, WNT signaling pathway and LRP1 were significantly enhanced by miR-640 inhibition.

CONCLUSIONS: These results suggested that miR-640 promotes KCs inflammation via restraining LRP 1 and WNT signaling pathway. But inhibiting miR-640 prevents inflammation damage and ameliorates ALI. MiR-640 inhibition may become a novel target for the therapy of ALI in the future.

Key Words:
MiR-640, Kuffer cells, Acute liver injury, LRP1.

Introduction

Acute liver injury is one of the common clinical diseases. Liver cells are stimulated by various factors that lead to a large number of degeneration and necrosis, which can engender shock, coagulation dysfunction, severe infection, hepatic encephalopathy, renal failure, etc.[1-3]. There are many etiologies leading to acute liver injury (ALI), such as drugs, virus infection, autoimmune liver disease, liver transplantation[4,5]. To explore effective liver protection therapy has always been the goal of many researchers. Liver injury involves many mechanisms, including immune response, inflammatory response, oxidative stress, endoplasmic reticulum stress, and apoptosis[6,7]. The role of immune response has been a hot topic of research. When liver injury occurs, the natural immune system in liver can be first activated by the breakdown products of damaged liver cells and exogenous antigens, such as macrophages, NK cells, and neutrophils[8]. Then, adaptive immune response activation, such as effector T cells, B cells and so on, produces corresponding effects. Accumulating studies[9,10] have verified that the expression of many genes and post-transcriptional modulation play a critical role after ALI. MicroRNA (miRNA) has gradually become an important post-transcriptional regulation mode because it can inhibit mRNA translation, which has attracted extensive attention[11]. MiRNAs, first discovered in Caenorhabditis elegans in 1993, are endogenous, 20-24 nucleotide long miRNAs[12]. In human body, miRNAs mainly rely on binding to the 3'UTR of target mRNAs to intensify the degradation of target mRNAs or inhibit their translation procedure, so as to interfere gene expression by mediating the post-transcriptional level and then regulate the functional level of cells, which is a common gene regulation mode in cells[13]. Besides, multiple miRNAs can regulate the same gene, and it is also possible that miRNAs can play a coefficient action in the post-transcriptional regulation of multiple genes[14]. Moreover, Song et al[15] confirmed that multiple miRNAs involved in ALI pathological mechanism. However, miR-

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640 effect to ALI is still unclear. In the present study, we sought to miR-640 effect and target on ALI, and through Kuppler cells (KCs) inflammation model and murine ALI model, we explored whether miR-640 overexpression and inhibition administration affected inflammation and hepatic function. Based on these preliminary outcomes, miR-640 inhibition application in progress of anti-ALI inflammation and the potential mechanism affecting LRP1.

Materials and Methods

Cells Culture

KCs were purchased from Beijing Zhongke Quality Inspection Biotechnology co., LTD (Beijing, China) and were cultured in Dulbecco's Modified Eagle's Medium and Ham's F-12 medium (DMEM/F12, KeyGEN, Nanjing, China) supplemented 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% Penicillin-Streptomycin. KCs were seeded in 5*5 cm_2 flask.

Transfection and Luciferase Assay

After the cell confluence to 90%, we transfected miR-640 mimic or miR-640 inhibitor (Sangon Biotech, Shanghai, China) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following manufacturer's instruction. The inflammatory KCs model is established by lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) stimulus for 24 h. KCs transfected with Luciferase-labeled miR-640 mimic or miR-640 inhibitor (15 ng) *via* adeno-associated virus (AAV) were isolated after undergoing 72 h transfection. Luciferase concentration in the transfected cells was detected using Luciferase assay system (Promega, Madison, WI, USA) abided by protocol.

Mice

Six to eight-week-old C57/B6J male mice (average weight 20 g) purchased from Fujian Medical University, were bred at the Fujian Medical University Animal Center. Mice were provided with available normal food and water. Six mice were housed in a cage with suitable temperature (22°C-25°C) and humidity (55%-65%), and 12 h light/dark cycle. Our study was approved by the Animal Ethics Committee of Fujian Medical University, and all researches were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the Fujian Medical University.

Acute Liver Injury and MiRNA Injection

The animals were randomly divided into four groups: control group (n=6); ALI group (n=6); control inhibitor group (n=6); miR-640 inhibitor group (n=6). Briefly, tail intravenous injections of miR-640 control inhibitor and miR-640 inhibitor were administered at 72 h before LPS induction. Next, 10 mg/kg LPS were treated mice to established ALI model through intraperitoneal injection.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from macrophages with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) directed by the manufacturer's protocol. Reverse transcription was conducted to synthesize cDNA using PrimeScript™ RT Master Mix (Applied Biosystems, Foster City, CA, USA). RT-PCR was conducted to quantify mRNA expression levels. Then, RT-PCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). 18S rRNA was used for normalization. Relative mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ methods. Primer sequences are listed as follows: MiR-640: Forward (5'>3'), GCCCCTGCAGAGCACTGCGG, Reverse (5'>3'), GGCCACCCGGCGGCCGGCAA; LRP1: Forward (5'>3'), GGACCGCTCTGATGAGTCTG, Reverse (5'>3'), CAGTCATTGTCATTGTCGCATCT; β-catenin: Forward (5'>3'), ATGACTCGAGCTCAGAGGGT, Reverse (5'>3'), ATTGCACGTGTGGCAAGTTC; EP300: Forward (5'>3'), TTGTGATCCGCCTCATTGCT, Reverse (5'>3'), CGACCATCCATCAGATCGCA; TNF-α: Forward (5'>3'), ATGTCTCAGCCTCTTCTCATTC, Reverse (5'>3'), GCTTGTCACTCGAATTTTGAGA; IL-1β: Forward (5'>3'), TGCTCATGTCCTCATCCTGGAAGG, Reverse (5'>3'), TCGCAGCAGCACATCAACAAGAG; IL-6: Forward (5'>3'), CTCCCAACAGACCTGTCTATAC, Reverse (5'>3'), CCATTGCACAACTCTTTTCTCA; 18S rRNA: Forward (5'>3'), GTTGGTTTTCGGAACTGAGGC, Reverse (5'>3'), GTCGGCATCGTTTATGGTCG. U6: Forward (5'>3'), CTCGCTTCGGCAGCACA, Re-verse (5'>3'), AACGCTTCACGAATTTGCGT; GAPDH: Forward (5'>3'), TGACTTCAACAGCGACACCCA, Re-verse (5'>3'), GGAGTGTTGGAGAAGTCATATTAC.

Western Blot (WB) Analysis

Liver tissue and HLMs were harvested in lysis buffer. Proteins were isolated using a Total

Protein Extraction Kit (Keygen, Nanjing, China) according to the manufacturer's instructions. Protein concentrations were measured with the Enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). After separation, transferring and blocking with 5% skim milk for 1 h at room temperature, proteins were incubated overnight with anti-LRP1 (Abcam, Cambridge, MA, USA, 1:1000), anti-caspase-3/8 (Abcam, Cambridge, MA, USA, 1:1000), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA, 1:2000). Washing with Tris-Buffered Saline and Tween-20 (TBST), the membrane was incubated with secondary antibody (YiFeiXue, Nanjing, China, 1:10000) for 1 h at room temperature. Proteins were visualized and detected using an enhanced chemiluminescence (ECL) system.

Flow Cytometry Analysis

The apoptosis was determined using the 70-AP101-30 Annexin V-FITC/PI Apoptosis Kit (MultiSciences, Hangzhou, China) based on the manufacturer's procedures. Liver cells were incubated with Annexin V-FITC and propidium iodide (PI) in away from light for 30 min. Apoptotic cells and viable cells were sorted using a fluorescence-activated cell sorting flow cytometer (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Histologic Staining

Hepatic tissue was fixated with 4% paraformaldehyde and dehydration was gotten through different concentration alcohol. Then, tissue was embedded into paraffin and cut into sections (4 μm). Hematoxylin-eosin staining was conducted using Hematoxylin and Eosin Staining Kit (Beyotime, Shanghai, China) following manufacturer's protocol.

Immumohistochemical (IHC) Staining

Hepatic tissue was obtained through fixation and dehydration, then it was embedded into paraffin and cut into sections. Following deparaffinization and rehydration, tissue section was conducted immunohistochemistry (IHC) with TNF-α (Abcam, Cambridge, MA, USA, 1:200) and IL-1β (Abcam, Cambridge, MA, USA, 1:300). Then, images were visualized using a microscope.

Immunofluorescence (IF)

KCs (1×105/ well) were obtained through fixation and then it was conducted IF with LRP1(Abcam, Cambridge, MA, USA, 1:500) overnight at 4°C, followed by Cy3-conjugated goat anti-rabbit IgG antibody (Abcam, Cambridge, MA, USA, 1:200) for 2 h at room temperature. Nuclei were counterstained with DAPI-Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Then, images were collected using a microscope.

Enzyme Linked Immunosorbent Assay

Serum was taken from mice. The cell medium and serum were centrifuged for 10 min and then the supernatant was collected. Standard product was added in a 96-well plate with different concentration successively. Then, the colorant was seeded into each well and the samples were incubated without light for 15 min. The termination solution was added to terminate the reaction and the absorbance (OD value) of each well was measured sequentially at 450 nm.

Statistical Analysis

Data were displayed as the means ± standard deviations. Comparison between the two groups was analyzed using Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Data were collected and assessed using GraphPad Prism (La Jolla, CA, USA). When $p<0.05$, differences were considered statistically significant.

Results

LRP1 Serves As a Target of MiR-640 Following ALI

First, we explored whether miR-640 changes in ALI after LPS instigation. Then, liver tissue was extracted into RNA to detect miR-640 level following ALI in mice within 3 days. The result showed that LPS-induced ALI triggered miR-640 elevation at post injury, especially the highest expression level of miR-640 was at one day post injury (Figure 1A). Then, LRP1 RNA level was measured, finding that LRP1 expression decreased at post-ALI and the lowest LRP1 RNA level was at one days post-ALI (Figure 1B). Hence, to verify whether miR-640 targets LRP1 to response to ALI, we utilized miR-640 mimic and miR-640 inhibitor into KCs and detected transfection level using Dual-Luciferase reporter, suggesting that miR-640 mimic overexpressed miR-640 level and miR-640 in-

hibitor repressed miR-640 level (Figure 1C). Moreover, qRT-PCR exhibited that miR-640 mimic down-regulated LRP1 RNA level and miR-640 inhibitor increased LRP1 expression (Figure 1D). Besides, LRP1 protein was reduced *via* miR-640 mimic but elevated LRP1 expression was in the miR-640 inhibitor group (Figure 1E). Therefore, the above results indicated that miR-640 interacted with LRP1 transcription at post ALI.

MiR-640 Regulates Inflammation of KCs Negatively Targeting LRP1 and WNT Pathway

Furtherly, we explored whether miR-640 expression difference affects KCs inflammation level. QRT-PCR displayed that tumor necrosis factor-alpha (TNF-α) interleukin 1 beta (IL-1β), and interleukin 6 (IL-6) remarkably increased following miR-640 mimic treatment, but miR-640 inhibitor down-regulated inflammatory factors transcription with LPS stimulation or not (Figure 2A-2C). Besides, ELISA on a variety of inflammatory factors including TNF-α, IL-1β, and IL-6 in cell free supernatant showed that miR-640 mimic slightly increased the expression of TNF-α, IL-1β, and IL-6, and miR-640 inhibitor suppressed the expression of TNF-α, IL-1β, and IL-6. After LPS activation, miR-640 mimic significantly promoted the release of TNF-α, IL-1β, and IL-6, while miR-640 inhibitor significantly restrained the levels of the inflammation factors (Figure 2D-2F). To understand the effect of miR-640 on the expression of LRP1 in KCs, we visualized the expression of LRP1 by immunofluorescence staining, and found that the expression of LRP1 following mimic treatment was significantly reduced while the expression of LRP1 was increased *via* inhibitor administration after KCs activation or not (Figure 2G). Moreover, we measured β-catenin and EP300 expression in RNA level, showing that mimic evidently declined β-catenin and EP300 level but inhibitor effectively up-regulated β-catenin and EP300 expression after LPS treatment or not (Figure 2H, 2I). So, the results indicated that miR-640 participated

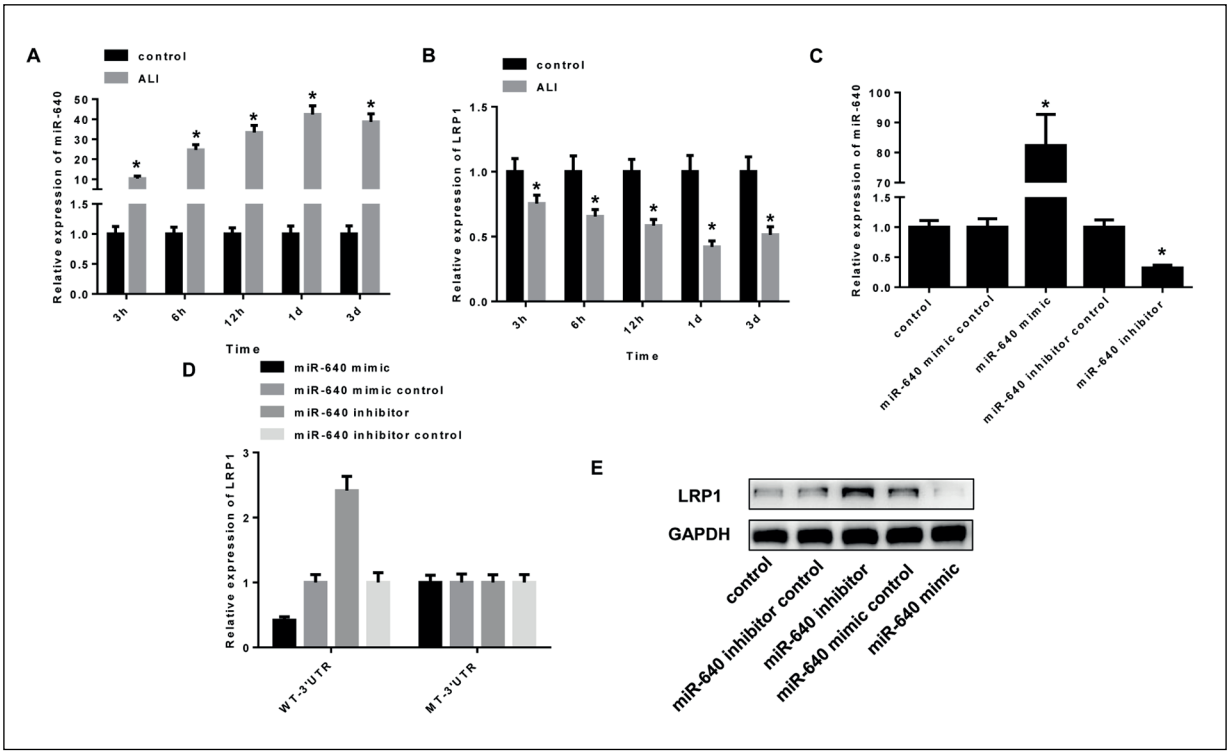

Figure 1. LRP1 serves as a target of miR-640 following ALI. **A,** Representative miR-640 RNA level at 3 h, 6 h, 12 h, 1 day, 3 days after ALI. **B,** Representative LRP1 RNA level at 3 h, 6 h, 12 h, 1 day, 3 days after ALI. **C,** Representative miR-640 RNA level transfected with miR-640 mimic control, miR-640 mimic, miR-640 inhibitor control, miR-640 inhibitor in KCs. **D,** Luciferase report of miR-640 mimic control, miR-640 mimic, miR-640 inhibitor control, miR-640 inhibitor to WT-3'UTR and MT-3'UTR LRP1 RNA. **E,** Representative LRP1 protein level transfected with miR-640 mimic control, miR-640 mimic, miR-640 inhibitor control, miR-640 inhibitor in KCs. "*" means *vs.* control group with statistical significance.

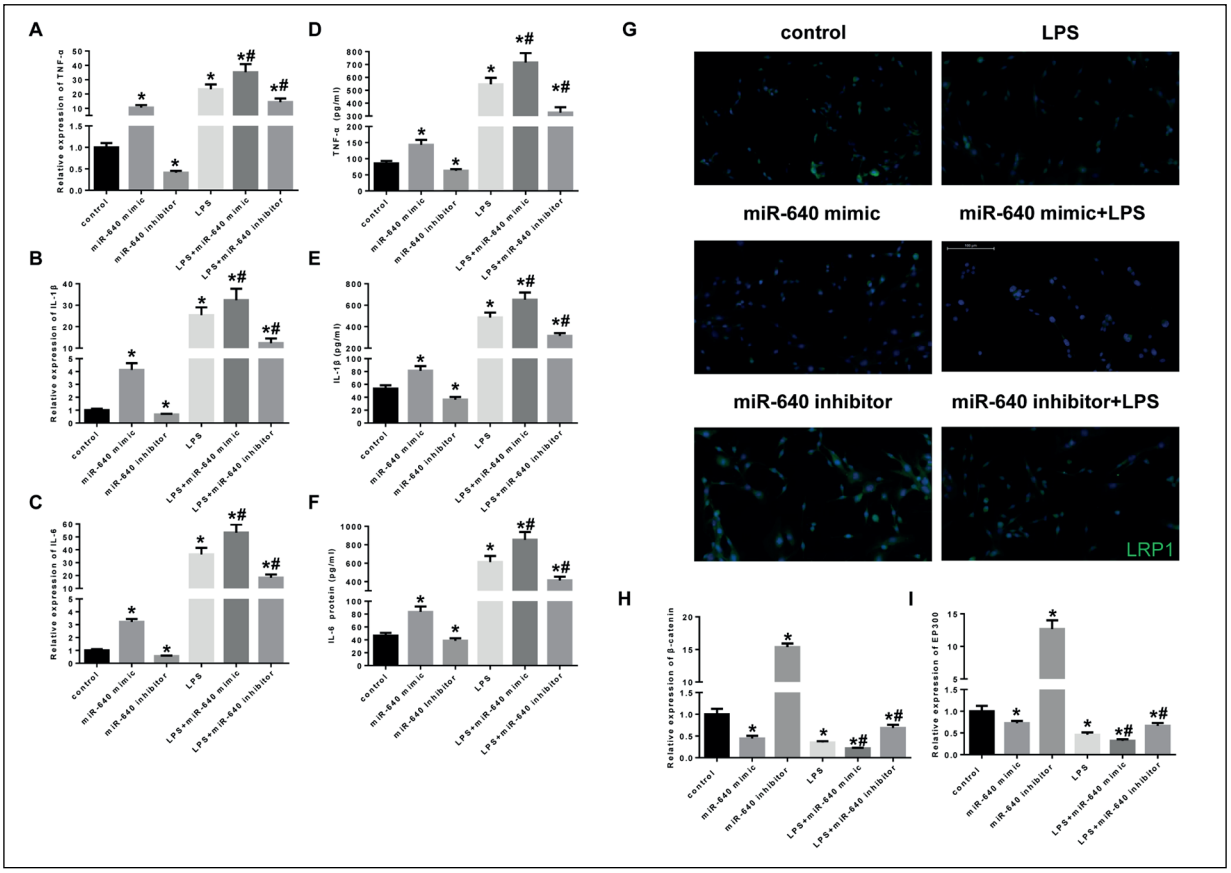

Figure 2. MiR-640 regulates inflammation of KCs negatively targeting LRP1 and WNT pathway. **A-C,** Representative RNA level of TNF-α, IL-1β, and IL-6 in the control, miR-640 mimic, miR-640 inhibitor, LPS, LPS+ miR-640 mimic and LPS+miR-640 inhibitor group. **D-E,** Representative ELISA of TNF-α, IL-1β and IL-6 in control, miR-640 mimic, miR-640 inhibitor, LPS, LPS+ miR-640 mimic and LPS+miR-640 inhibitor group. **G,** Representative immunofluorescence of LRP1 in control, miR-640 mimic, miR-640 inhibitor, LPS, LPS+ miR-640 mimic, and LPS+miR-640 inhibitor group (magnification: 200×). **H-I,** Representative RNA level of β-catenin and EP300 in the control, miR-640 mimic, miR-640 inhibitor, LPS, LPS+ miR-640 mimic and LPS+miR-640 inhibitor group. "*" means *vs.* control group with statistical significance. "#" means *vs.* LPS group with statistical significance.

KCs inflammation by down-regulating LRP1 and WNT pathway.

MiR-640 Inhibition Alleviates ALI-Induced Inflammation and Apoptosis In Mice

We evaluated the therapeutic effect of miR-640 inhibition on ALI in mice and achieved miR-640 down-regulation through transfection with miR-640 inhibitor. Western blotting showed that the protein level of LRP1 increased following miR-640 inhibitor transfection, while the protein level of LRP1 decreased significantly after ALI, miR-640 inhibitor prevented the decrease of LRP1 after ALI (Figure 3A). Immunohistochemistry showed that TNF-α and IL-1β did not change significantly after miR-640 inhibitor administration, but the expression of TNF-α and IL-1β increased significantly after ALI, and the miR-640 inhibitor had a significant restriction effect on the expression of TNF-α and IL-1β (Figure 3B). To confirm whether miR-640 inhibition restraining inflammation affects the degree of apoptosis in tissues, we conducted flow cytometry assay of apoptotic cells in liver tissues, and found that the level of apoptosis in liver tissues increased significantly after ALI, while miR-640 inhibitor treatment significantly ameliorated the degree of apoptosis (Figure 3C). Furthermore, apoptosis related factors we detected by Western blotting, showing that the expression of caspase 3 and caspase 8 was significantly increased in ALI group. However, underwent 640 inhibition, the expression levels of caspase 3 and caspase 8 were significantly suppressed, which may lead to the decreased apoptosis level (Figure 3D). There-

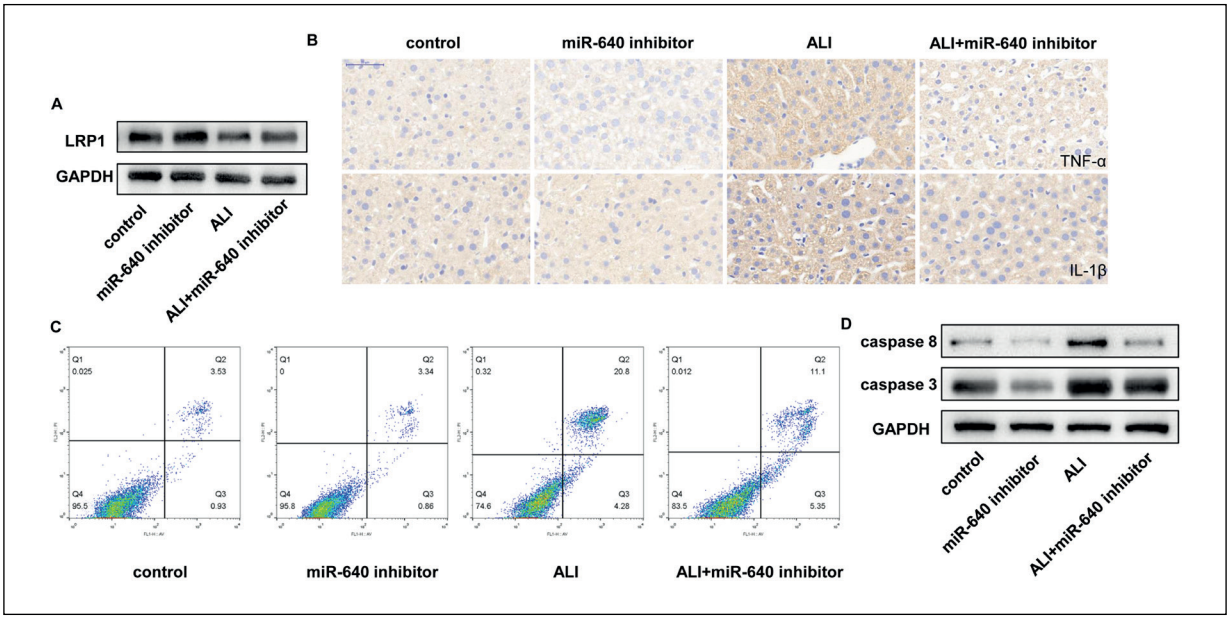

Figure 3. MiR-640 inhibition alleviates ALI-induced inflammation and apoptosis. **A,** Representative Western blotting of LRP1 in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group. **B,** Representative IHC of TNF-α and IL-1β in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group (magnification: 200×). **C,** Representative flow cytometry assay of apoptosis in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group. **D,** Representative Western blotting of caspase 3 and 8 in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

fore, the above results suggested that miR-640 inhibitor decreased ALI-induced inflammation and apoptosis.

MiR-640 Inhibition Improves Hepatic Histology and Function In ALI

We examined the treatment of miR-640 inhibitor on hepatic histology and function following ALI. HE staining showed that hepatic tissue in control and miR-640 inhibitor group, which has intact cell morphology, prominent nucleus, no inflammation or necrosis. However, LPS provoked diffuse lesion and necrosis of hepatic cells, resulting in hemorrhage, swelling, necrosis, and inflammatory cell infiltration. Under the administration of miR-640 inhibitor, LPS-mediated alteration in hepatic pathomorphology was significantly corrected (Figure 4A). In addition, LPS induction elevated serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) concentration, and total bilirubin (TBIL) level, but restraining miR-640 treatment reduced serum ALT, AST concentration and TBIL level resulted from LPS instigation (Figure 4B, 4C). Therefore, miR-640 inhibition ameliorates hepatic histology and function in ALI.

Discussion

Liver injury is a process of inflammatory development, which can produce many cytokines[16]. As intrinsic macrophages in liver, KCs participate in the inflammatory response of liver. LPS can activate KCs straightway and trigger inflammatory effect to provoke durative injury[17]. However, various miRNAs play an important regulatory role in the process of inflammation[18]. Earlier studies[19,20] have reported that miR-146a served as an inflammation-associated microRNA in human temporal lobe epilepsy[19] and miR-223involved in infection, inflammation, and cancer[20]. Moreover, elevated miR-155 promotes inflammation in cystic fibrosis[21]. Consistently, miR-640, as an inflammatory actuator, aggravates intervertebral disc degeneration through *via* NF-κB and WNT signaling pathway[22]. In our study, we hypothesized that miR-640 in ALI may also play a role in improving damage effects during inflammation by inhibiting WNT signaling pathway and inflammation-related factors. Therefore, we measured miR-640 and LRP1 levels in ALI *via* LPS stimulation within

3 days and found that the expression of miR-640 was significantly elevated. Oppositely, LRP1 level was decreased. We further confirmed the overexpressed or inhibitory effect of miR-640 in KCs and found that miR-640 overexpression up-regulated the expression of inflammation by inhibiting LRP1 and WNT pathway. Multiple studies have proved that LRP1 and WNT pathway are involved in the mediation of inflammatory response. In our research, we proved that lower expression of WNT pathway after inflammatory response and miR-640 to inflammatory response aggravation of ALI is realized *via* down-regulating WNT pathway and inhibiting LRP1. Hence, the concentrations of pro-inflammatory factors and chemokines such as TNF-α, IL-1β, and IL-6 released from miR-640 mimic treated KCs were significantly increased. Besides, LPS-induced ALI disease model was established in mice, and miR-640 inhibitor was applied to evaluate the efficacy. *In vivo*, we reconfirmed that miR-640, as a LRP1 inhibitor, down-regulated WNT pathway activation and played a pro-inflammatory role. Of note, we found that miR-640 inhibition blocked the inflammation-mediated elevation of TNF-α, IL-1β in liver, suggesting that miR-640 inhibition also reduced the infiltration of peripheral inflammatory cells into liver tissue. LPS stimulates KCs to secrete large amounts of TNF-α, which directly mediates apoptosis by binding to TNF-α receptor 1, and TNF-α activates caspase 8 in liver cells, leading to Bax oligomerization and insertion into mitochondria, initiating apoptosis of the mitochondrial pathway. Therefore, a group number of apoptotic cells can be detected in ALI model. However, miR-640 inhibition could effectively decrease the level of TNF-α and down-regulate the expression of caspase3 and 8. Therefore, we found that the apoptosis level in liver tissue was significantly improved following miR-640 inhibition treatment in ALI. Thus, miR-640 inhibition ultimately improves liver function and protects liver tissue structure. Therefore, miR-640 inhibition finally lowered the serum content of ALT, AST, and TBIL after ALI and reduced the area of tissue damage caused by inflammation in liver tissues, thus improving liver function and protecting liver tissue structure. To sum up, in the present study, we

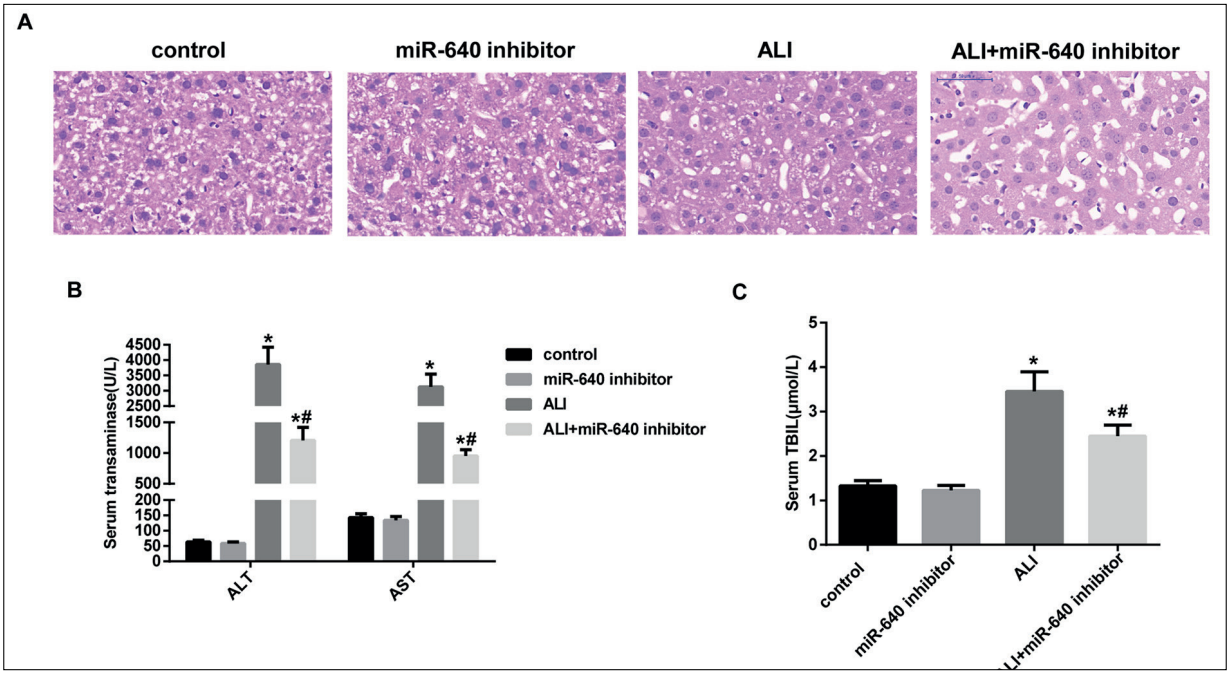

Figure 4. MiR-640 inhibition improves hepatic histology and function in ALI. **A,** Representative HE staining in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group (magnification: 200×). **B,** Representative serum ALT and AST level in the control, miR-640 inhibitor, ALI and ALI+ miR-640 inhibitor group **C,** Representative serum TBIL detection in the control, ALI and GL group. "*" means *vs.* control group with statistical significance. "#" means *vs.* ALI group with statistical significance.

proved miR-640 inhibition mediated liver protection in LPS-induced ALI, miR-640 inhibition got by inhibiting LRP1 and regulating WNT pathway to play an antagonism of inflammatory infiltration, which triggered by macrophages in the liver. Moreover, miR-640 inhibition mitigated the levels cell apoptosis in liver tissue, so as to improve hepatic function and prevent excessive damage to hepatic structure.

Conclusions

These results indicated that miR-640 promotes KCs inflammation *via* restraining LRP 1 and WNT signaling pathway. Inhibiting miR-640 prevents inflammation damage and ameliorates ALI. MiR-640 inhibition may become a novel target for the therapy of ALI in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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