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ORIGINAL ARTICLE

MicroRNA-124a/Sirtuin 1 pathway inhibits autophagy to promote hepatocyte apoptosis in acute-on-chronic liver failure

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Abstract

Background The roles of microRNAs in the regulation of autophagy and apoptosis in hepatic cells suggest that they may serve as novel biomarkers or therapeutic targets for various liver injuries. In this study, we aim to analyze whether miR-124a regulates autophagy and apoptosis in hepatic cells, particularly in acute-on-chronic liver failure (ACLF).

Materials and methods The plasma and liver tissues from the healthy control (HC) and ACLF patients were included. Moreover, LO2 cells were used to perform in vitro experiments. To measure hepatocyte apoptosis, a TUNEL kit was used. LPS, over-expression or knockdown, 3-methyladenine (3-MA) were used in vitro experiments. The expression levels of the autophagy related proteins (Beclin-1 and LC3), anti-apoptotic proteins (BAX and Bcl-2), Sirtuin 1 (SIRT1), and miR-124a were assessed using western blotting, ELISA, and qRT-PCR.

Results ACLF patients had significantly decreased expressions of SIRT1, Bcl-2, LC3, and Beclin-1 and significant upregulation of miR-124a and BAX in both plasma and liver tissues in comparison with the HC group. miR-124a was inversely correlated with autophagy markers and SIRT1, but positively correlated with apoptosis. Upon exposure to LPS, the levels of BAX and miR-124a were notably elevated, while Beclin-1, LC3, SIRT1, and Bcl-2 were notably downregulated in LO2 cells. These changes were further exaggerated in the presence of the miR-124a mimic and EX-527 compared to the miR-124a inhibitor and SRT1720 groups. Co-transfection of miR-124a inhibitor was able to partly counteract the pro-apoptotic effects of the autophagy inhibitor 3-MA.

Conclusion miR-124a downregulates SIRT1, thereby suppressing hepatocyte autophagy and consequently inducing apoptosis.

Keywords miR-124a · Sirtuin 1 · Autophagy · Apoptosis · Acute-on-chronic liver failure

Introduction

ACLF represents a high health risk disease characterized by the rapid deterioration of liver function in patients with chronic liver disease, who are either in an undiagnosed or diagnosed state of cirrhosis. It is associated with jaundice, ascites, coagulopathy, hepatic encephalopathy, and extrahepatic organ failure [1, 2]. The majority of general or global causes of ACLF onset are believed to be reactivation of chronic viral hepatitis, primarily hepatitis B, and alcohol abuse [1, 2]. Increasing evidence highlights the systemic dysregulation of inflammatory responses and hepatocyte death, particularly apoptosis, as key mediators in the progression of ACLF. Despite significant advancements in artificial liver support and antiviral therapy, ACLF remains a severe clinical issue with high mortality rates, often exceeding 28–90 days post-onset [3]. Multiple clinical challenges arise from our incomplete understanding of the diverse pathways and mechanisms involved in ACLF progression. Thus, effective treatments and interventions that prevent the progression of chronic liver disease in the absence of acute exacerbation could reduce the incidence of ACLF.

Autophagy is a cellular housekeeping activity that maintains homeostasis and cell renewal in eukaryotic cells by transporting dysfunctional or surplus proteins via vesicles

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or lysosomes [4]. Dysfunction in autophagy has increasingly become an attractive topic in various pathological mechanisms, ranging from metabolic disorders and inflammation to cell death [5, 6]. The autophagic process is regulated by Sirtuin 1 (Silent Information Regulator 1, SIRT1), which modulates this signaling pathway by influencing certain proteins and pathways, thereby guiding immune and inflammatory responses [7, 8]. Growing evidence suggests that SIRT1, a deacetylase, promotes the activation of autophagy and prevents apoptosis under various pathological conditions [9–11]. Notably, there are instances where autophagy and autophagy-related proteins lead to necrosis or apoptosis through various pro-death processes [12, 13]. Importantly, much work remains to be done regarding the role of autophagy in ACLF, necessitating further research to elucidate the interplay between autophagy and apoptosis in this context.

MicroRNAs (miRNAs) can bind to the 3'-untranslated region (UTR) of target messenger RNAs (mRNAs), inhibiting translation and/or promoting mRNA degradation. MiRNAs control numerous biological processes, such as cell migration, proliferation, programmed autophagy, and cell death [14–16]. The presence of miRNAs has been confirmed in various bodily fluids, including serum, plasma, urine, cerebrospinal fluid, and tears. As stable biomarkers in serum and plasma, several miRNAs are considered promising for diagnosing and predicting the progression of various liver diseases [17]. MiR-124, predominantly found in the brain, plays a crucial role in central nervous system functions and regulates metastasis and tumorigenesis by targeting oncogenic proteins [14, 16, 18–20]. Irregular expression levels of miR-124 are closely associated with hepatitis B virus infection and liver failure [21]. Studies have also indicated that miR-124 has multifaceted roles in different disease mechanisms and stages of autophagy and apoptosis [6, 14, 19, 20]. However, the impact of miR-124 on autophagy in patients with ACLF has received limited attention; thus, elucidating miR-124's effect on autophagy in ACLF is necessary and meaningful. SIRT1 is one of the target genes regulated by miR-124. Additionally, the miR-124/SIRT1 axis is implicated in various diseases [22, 23]. Given the significant roles of SIRT1 and miR-124 in liver injury, this study aims to clarify their functions in regulating autophagy and apoptosis processes, potentially providing a theoretical basis for understanding the pathophysiology and progression of ACLF.

In this study, liver tissue samples and plasma were collected from patients with ACLF. Lipopolysaccharide (LPS)-induced LO2 cells were used as an in vitro model for hepatotoxicity and apoptosis. MiR-124a mimics and inhibitors were employed, along with or without the SIRT1 activator (SRT1720) and the SIRT1-specific inhibitor (EX527), as well as 3-methyladenine, a specific autophagy inhibitor. The correlation between miR-124a and SIRT1, autophagy-related proteins, and apoptosis-related proteins in patients was established to investigate the association among SIRT1, miR-124a, apoptosis, and autophagy in ACLF. Generally, these findings could be of significant importance in elucidating the mechanisms and pathways of disease progression, potentially paving the way for the development of new drugs or attractive targets in the future clinical management of ACLF.

Materials and methods

Clinical samples

Our study included 40 participants from the Third Affiliated Hospital of Hebei University Medical College, covering the period from November 2016 to February 2019. Patients with ACLF were recruited based on the diagnostic and management criteria established by the American Association for the Study of Liver Diseases (AASLD). All patients provided informed consent prior to enrollment, and written consent confirmation was recorded for each patient. The study population comprised 20 healthy controls (HC) and 20 ACLF patients, among whom 5 underwent liver transplantation. For our experimental procedures, liver tissue samples were collected from transplantation surgeries, ensuring informed consent was obtained from the five ACLF patients who received transplants as well as from the five donors or their officially designated representatives.

Blood sample collection and biochemical parameters

Peripheral blood samples were collected from patients and healthy individuals via venipuncture and placed into sodium heparin tubes supplied by BD (Franklin Lakes, NJ, USA). The blood samples were immediately centrifuged at 2500 rpm/min for 10 min. Aliquots of plasma samples were transferred into screw-cap cryogenic vials from Corning (NY, USA), placed in a biosafety cabinet, and stored at -80 °C for further analysis. To maintain the integrity of the serum samples, they were promptly frozen at -80 °C shortly after processing to minimize the risk of degradation. All samples were aliquoted to avoid repeated freeze-thaw cycles. Among other tests, clinical chemistry studies of the serum included assessments of liver function, albumin, aspartate transaminase, serum alanine transaminase, bilirubin, and prothrombin time. All these parameters were analyzed using an automated biochemical analyzer at the Third Affiliated Hospital Medical School of Hebei University.

ELISA assay

The plasma levels of SIRT1, BCL-2, BAX, Beclin-1, and LC3 in the ACLF patients were measured using human SIRT1 ELISA kits (Abclone, Seoul, Korea) and LC3, Beclin-1, BCL-2, and BAX ELISA kits (Lianke Biotech, Hangzhou, China).

Hematoxylin and eosin (HE) staining

All liver tissue specimens were immersed in a 4% formaldehyde solution and fixed overnight at room temperature, followed by embedding in paraffin. Subsequently, 3.5 μm thick sections were created, mounted on slides, and processed through a series of steps, including deparaffinization in xylene and rehydration through graded alcohols to water. The sections were stained with H&E according to standard protocols. Initially, after deparaffinization and rehydration, the 3.5 μm sections were briefly stained with hematoxylin (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) for 1–3 min, treated with 1% acidic ethanol for two seconds, and then rinsed with tap water. Subsequently, the sections were stained with eosin (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) for 2 min and washed with running water. The slides were then dehydrated, cleared, and mounted using alcohol, xylene, and neutral balsam. Finally, the H&E-stained sections were examined under a light microscope to evaluate their staining characteristics.

Apoptosis detection

As previously described, the samples underwent H&E staining pre-treatment. Apoptosis was detected using the TUNEL apoptosis detection kit (Solaibao, Beijing, China). The experimental steps are summarized as follows. Initially, the sections were placed in PBS buffer solution with a pH of 7.4 and then treated with proteinase K at 37 °C for 20 min. Subsequently, they were rinsed in a decolorizing shaker for three cycles, each lasting 5 min. A labeling solution containing labeled nucleotides and TdT enzyme was then applied, and the sections were incubated in the dark at 37 °C for 60 min. Slides immersed only in the labeling solution served as negative controls. To stain the cell nuclei, DAPI was used for a 10-minute incubation. The fluorescently labeled sections were examined, and their images were captured using a Nikon stereomicroscope (Tokyo, Japan).

Cell culture and treatment

The LO2 cell line, derived from human hepatocytes, was obtained from Procell Life Science & Technology Co., Ltd. (Wuhan, China). The cells were cultured in DMEM containing 4.5 g/L glucose, sourced from Gibco (Waltham, MA, USA), supplemented with 10% heat-inactivated FBS from GEMINI (USA) and 1% penicillin/streptomycin from Biological Industries (BI). Cultures were maintained in a humidified incubator at 37 °C with an atmosphere of 95% air and 5% CO_2. To establish an in vitro model of hepatocyte injury, cells were treated with varying concentrations of LPS (up to 30 μg/ml) for 0, 6, 12, and 24 h. For apoptosis model studies, LPS was used at a concentration of 10 μg/ml. SRT1720 and EX527, sourced from MCE (Monmouth Junction, NJ, USA), as well as 3-methyladenine (3-MA), were dissolved in DMSO and added to serum-free media at appropriate concentrations.

Cytotoxicity assay

To assess the short-term effects of LPS on cell viability, a CCK-8 assay was performed. Briefly, LO2 cells were seeded into sterile 96-well plates at the density of 5×10^3 cells per well, and incubated for 24 h. The cells were treated with LPS (0, 0.1, 1, 5, 10, 20, and 30 μg/ml) or vehicle control for 0, 6, 12, and 24 h at 37℃. After 2 ho of the incubation, 10 μL of CCK-8 solution (MedChemExpress, Shanghai, China) was added into all wells. Absorbance was measured at 450 nm.

Cell transfection

Upon reaching 50–60% confluence, either miR-124-3p inhibitor, miR-124-3p mimic (MH10691, Ambion, USA), or their respective negative controls-micrOFF™ inhibitor negative control (miR02101-1-5, RiboBio, China) and micrON™ mimic negative control (miR01101-1-5, RiboBio, China)-were transfected into LO2 cells. Lipofectamine® RNAiMAX Reagent (Invitrogen, USA) was used for transfection. The primer sequence for inhibitor is 5'-GGCAUUCACCGCGUGCCUUA-3'. The primer sequence for miR-124-3p mimic is 5'-CAUUCACCGCGUGCCUUATT-3'. The efficiency of miR-124-3p overexpression and knockdown was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The cells that were successfully transfected were then subjected to further downstream experimental procedures.

qRT-PCR

The RNA extraction was done using Qiagen's miRNeasy Mini Kit for liver tissues and LO2 cells, with the miRNeasy Serum/Plasma Kit (Hilden, Germany) for plasma. The reverse transcription from RNA into cDNA was done with the PrimeScript RT reagent Kit by Takara (Dalian, China),

and by utilizing Qiagen's miScript II RT Kit. Synthesis of the miR-124a cDNA was performed with Qiagen's PrimeScript RT reagent Kit. The miR-124a expression level was assessed and quantified utilizing Qiagen's miScript SYBR Green PCR kit, human miR-124a primers, along with C. elegans synthetic miR-39 (cel-miR-39) used as normalization control. mRNA analysis involved cDNA synthesis by Qiagen's miScript II RT Kit, followed by qRT-PCR using the QuantiNova SYBR Green PCR Kit. The endogenous controls for mRNA and miRNA expression were β-Actin from Invitrogen and U6 from Qiagen. All qPCR reactions were performed in triplicates. Relative expression levels of miR-124a and SIRT1 were calculated using the $2^{-\Delta\Delta Ct}$ method on an ABI PRISM 7,500 instrument according to the manufacturer's protocols for PCR amplification and analysis. The primer sequences used for miRNA and mRNA expression were as follows: SIRT1: Forward: 5'-AAGTTGACTGTGAAGCTGTACG-3'. Reverse: 5'-TG CTACTGGTCTTACTTTGAGGG-3'; β-Actin: Forward 5'-GTCACCTTCACCGTTCCAGTTTT −3'. Reverse 5'-CTTAGTTGCSGTTACACCCTTTCTT-3'.

Western blot

Liver tissues and LO2 cells were lysed using RIPA buffer along with protease inhibitors. The protein concentration was balanced among different samples before separation using 8% or 12% SDS-PAGE, followed by transfer to nitrocellulose membranes. After 2 h of blocking with 5% non-fat milk, the membranes were incubated overnight at 4 °C with the primary antibodies, including mouse monoclonal anti-SIRT1 (Abcam, Cambridge, UK) rabbit polyclonal antibodies for Beclin-1, LC3, Bcl-2, and BAX (Cell Signaling Technology, USA), and anti-beta-actin (Abcam). After washing, membranes were incubated for 2 h at room temperature with HRP-conjugated goat anti-rabbit or anti-mouse IgG (Abcam).

Table 1 Demographic and clinical characteristics in all enrolled subjects

Category	HC ($N=20$)	ACLF ($N=20$)	P value
Age (years)	46.84 ± 11.00	48.40 ± 12.17	>0.05
Gender, n (male/female)	20 (10/10)	20 (10/10)	1.00
ALT (U/L)	19.5 (16-23.75)	415 (343.5–469)	<0.01
AST (U/L)	22.50 (18–29)	405.5 (211-538.25)	<0.01
TBIL (μmol/L)	7.85 (6.43–9.32)	331 (247.13-369.71)	<0.01
DBIL (μmol/L)	4.88 (4.30–5.94)	189.65 (153.13-246.52)	<0.01

HC, healthy controls; ACLF, acute-on-chronic liver failure patients; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin

Annexin V-FITC/PI dual staining

After all the respective treatments, cells were rinsed and stained with the provided Annexin V-FITC/PI Apoptosis Detection Kit by BD Pharmingen (San Jose, CA, USA), following the manufacturer's instructions. Then the cells were analyzed through a flow cytometer of Beckman Coulter in Miami (FL, USA) to detect the type of apoptotic cells. The distribution of cells was determined as Annexin V+/PI+ (in a late phase of apoptosis) versus Annexin V+/PI- (indicating the early phase of apoptosis). The fraction of apoptotic cells was calculated with the help of FlowJo software.

Statistical analysis

Data are expressed as mean ± standard deviations (SD) or as medians with interquartile ranges. Statistical analysis was performed by using SPSS software package version 21.0. Comparisons between groups were performed by one-way ANOVA or Student's t-tests, respectively. The relationships between pairs of variables were measured using the Spearman rank correlation coefficient or the Pearson product-moment correlation coefficient. All comparison was performed using two-tailed tests with statistical significance set at a P-value less than 0.05.

Results

Patient characteristics of all enrolled subjects

Forty individuals constituted the study cohort, with 20 ACLF patients and 20 HC. There were no significant differences between groups concerning the ages or gender composition. The age in the ACLF group was 55.2 ± 6.4 years, whereas in the HC was 56.1 ± 5.9 years. ACLF group had significantly elevated levels of AST, ALT, DBIL, and TBIL and significantly reduced PTA compared to HC group ($P<0.01$). Table 1 demonstrates the clinical characteristics of the subjects attending the study.

Autophagy inhibition and apoptosis increases in the ACLF patients

H&E staining showed that the HC group had an orderly liver structure, and normal morphology, and the liver structure in the ACLF exhibited a mass of vacuoles. Loss of lobular structure of the liver tissue and liver cell disintegration with massive/submassive necrosis as the normal structure lost and apoptosis, cholestasis, and inflammatory cells penetrated (Fig. 1A). Based on reiteration of current research observations, LC3 and Beclin-1 had been chosen to assess

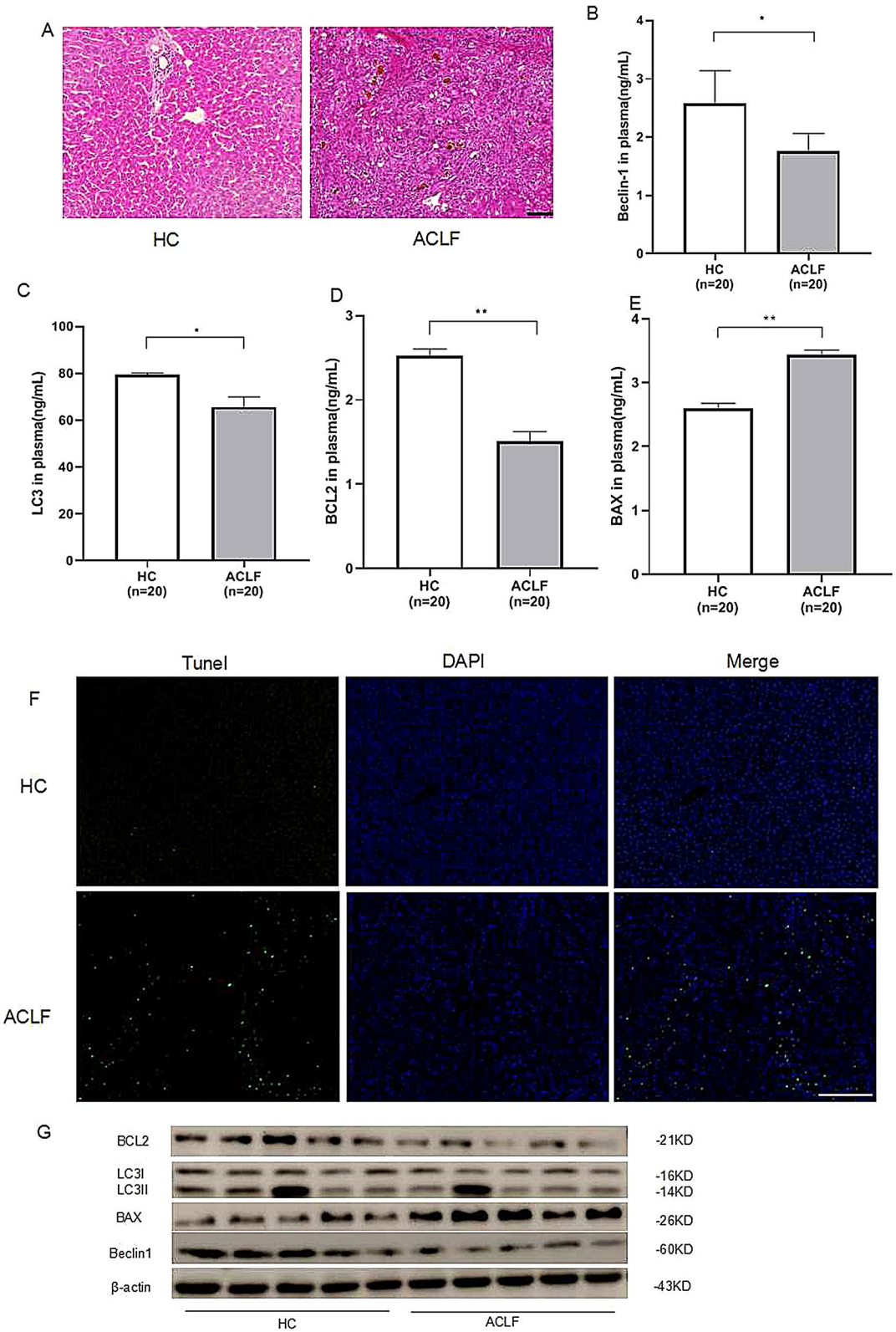
A
HC
ACLF
B
Beclin-1 in plasma(ng/mL)
HC (n=20)
ACLF (n=20)
C
LC3 in plasma(ng/mL)
D
BCL2 in plasma(ng/mL)
E
BAX in plasma(ng/mL)
Tunel
DAPI
Merge
F
HC
ACLF
G
BCL2
LC3I
LC3II
BAX
Beclin1
β-actin
-21KD
-16KD
-14KD
-26KD
-60KD
-43KD

Fig. 1 Significantly decreased autophagy level and increased cell apoptosis in the plasma and liver tissue samples of ACLF patients. (**A**) Pathological changes of liver tissues in the HC and ACLF groups detected by H&E staining (bar = 100 μm). The plasma levels of pro-apoptotic protein Bax (**B**), anti-apoptotic protein Bcl-2 (**C**), autophagy-related protein Beclin-1 (**D**), and autophagy-related protein LC3 (**E**) in the HC and ACLF groups determined by ELISA assay. (**F**) TUNEL assay was performed to detect the cellular apoptosis in liver tissues in the HC and ACLF groups (green, TUNEL-positive hepatocytes; blue, DAPI; bar = 200 μm). (**G**) The protein expression levels of Bax, Bcl-2, Beclin-1, and LC3 in liver tissues in the HC and ACLF groups were evaluated by western blot. β-Actin were used as a reference gene. * indicates $P<0.05$; ** indicates $P<0.01$

autophagy level in ACLF group. The ELISA and western blot analyses show that the protein levels of Beclin-1 and LC3 in plasma and liver tissues in the ACLF group were much lower compared with the HC group ($P<0.01$, Fig. 1B, C and G). These findings indicate that autophagy is suppressed in ACLF.

Plasma and liver tissue levels of BCL-2 were much lower in the ACLF group than in the HC group, but BAX levels were higher ($P<0.05$, Fig. 1D, E and G). TUNEL staining of liver tissue indicated that the percentage of TUNEL-positive cells was significantly greater in the ACLF group than in the HC group ($P<0.05$, Fig. 1F). Taken together, these results show suppression of autophagy with heightened apoptosis in ACLF patients.

Expression of miR-124a and SIRT1 in ACLF patients and their correlation analysis

qRT-PCR revealed that the miR-124a expression levels in liver tissues and plasma of ACLF patients were significantly higher than the HC group ($P<0.01$, Fig. 2A and B). However, the ACLF group had significantly lower SIRT1 levels than the HC group ($P<0.01$, Fig. 2 C and D). Moreover, the levels of miR-124a were negatively associated with SIRT1

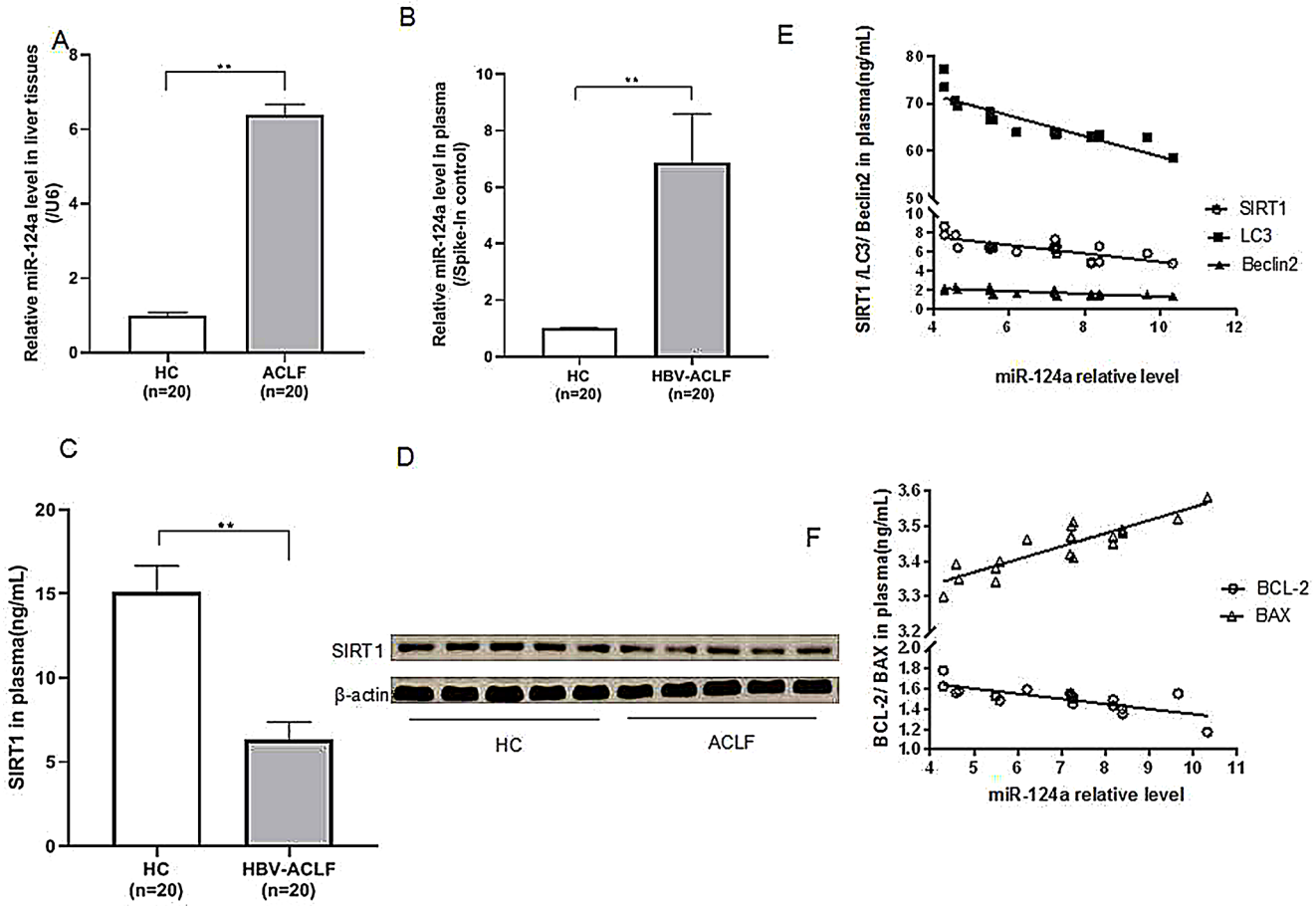

Fig. 2 A positive relationship between miR-124a and apoptosis related factors in ACLF, and a negative relationship of miR-124a with autophagy-related proteins and SIRT1, respectively. (**A** and **B**) The relative expression levels of miR-124a in plasma and liver tissues in the HC and ACLF groups determined by RT-PCR. (**C**) Plasma SIRT1 concentrations in the HC and ACLF groups detected by ELISA. (**D**) The SIRT1 protein expression levels in the liver tissues in the HC and ACLF groups detected by western blot. (**E**) Relationship of miR-124a with SIRT1, Beclin-1 and LC3 expressions in plasma of ACLF patients (Spearman's correlation test, $N=20$). (**F**) Relationship of miR-124a with Bcl-2 and BAX expression levels in plasma of ACLF patients (Spearman's correlation test, $N=20$). U6 was used as a reference. ** indicates $P<0.01$

expression in the ACLF group ($P<0.01$, Fig. 2E), with an R2 value of 0.5553. The levels of Beclin-1 and LC3 in plasma negatively correlated with plasma miR-124a levels which showed P-values below 0.01 and correlation coefficients of 0.7729 and 0.6384 (Fig. 2E). Additionally, correlation analysis showed that miR-124a expression was positively correlated with BAX levels but negatively correlated with Bcl-2 levels ($P<0.01$, Fig. 2F), with R2 values of 0.7821 for BAX and 0.5467 for Bcl-2. The findings suggest that miR-124a is negatively associated with SIRT1 and autophagy, but positively associated with apoptosis in ACLF patients.

LPS-induced autophagy inhibition and increased apoptosis in LO2 cells

To establish an in vitro model of hepatocyte injury, LO2 cells were treated with LPS (0, 0.1, 1, 5, 10, 20, 30 μg/ml) for 24 h. CCK-8 assay indicated that 10 μg/ml LPS was optimal for subsequent experiments ($P<0.05$, Fig. 3A). The viability of LO2 cells reduced progressively with further exposure to LPS and was lowest at the 24-hour mark ($P<0.05$, Fig. 3B). Flow cytometry showed that LPS-treated cells had a significantly higher apoptotic rate than control cells ($P<0.01$, Fig. 3C). Western blot showed that, at 6 h after 10 μg/ml LPS, the LC3II/I ratio and Beclin-1 expression were initially increased but significantly decreased at 24 h (Fig. 3D). This confirmed that the LPS-induced hepatocyte injury model was successfully established, showing an inhibition of autophagy and increased apoptosis.

LPS elevated miR-124a levels while simultaneously reducing SIRT1 expression

The miR-124a concentration in LO2 cells was significantly increased following an incubation of 10 μg/ml for 24 h than the control group ($P<0.01$, Fig. 4A). In contrast, SIRT1expression level was also notably decreased with LPS treatment ($P<0.01$, Fig. 4B, C).

miR-124a suppressed autophagy by targeting SIRT1

Introduction of a miR-124a mimic into LO2 cells notably increased miR-124a levels and sharply downregulated the levels of Beclin-1, LC3, and SIRT1 compared to the control

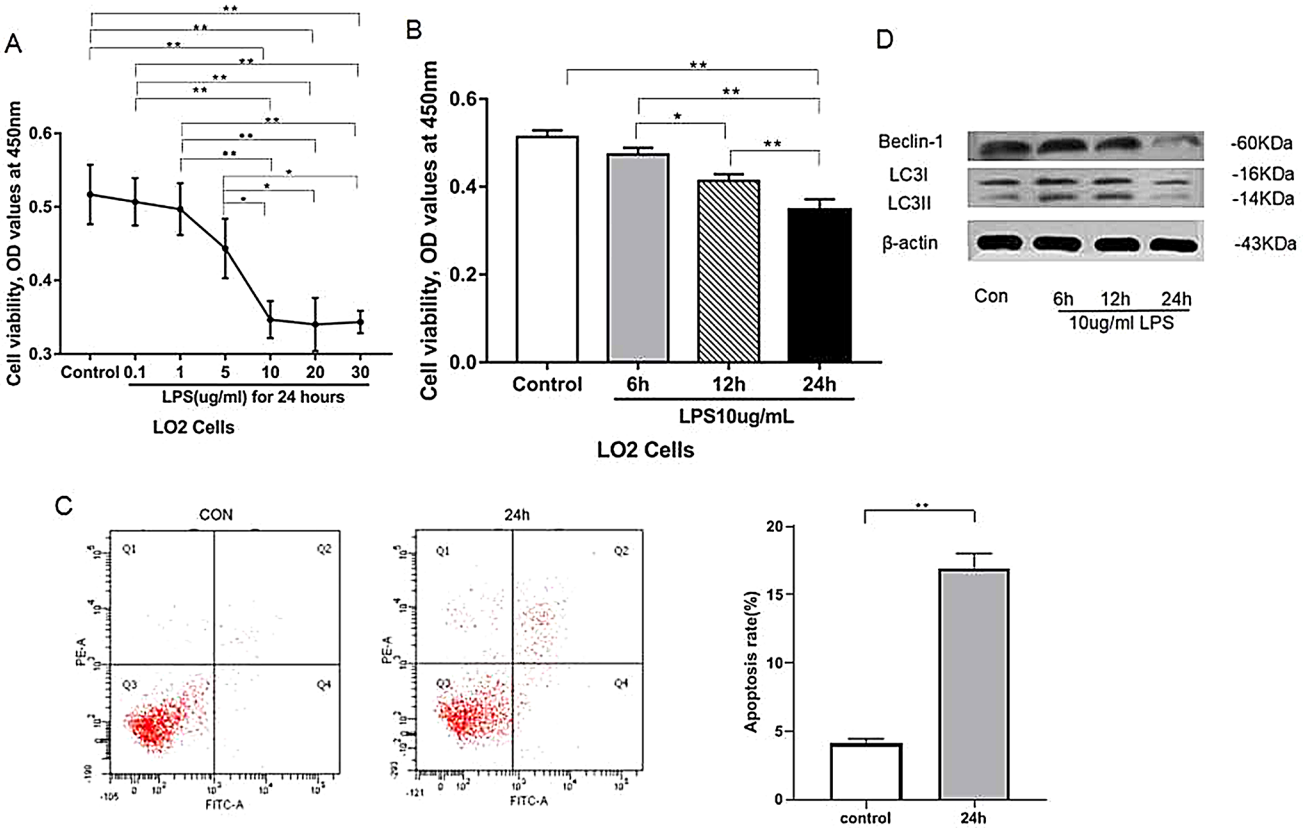

Fig. 3 LPS-mediated downregulation of cell viability and autophagy in LO2 cells. (**A**) Viability of LO2 cells was determined by the CCK8 assay after 24 h of incubation with LPS (0, 0.1, 1, 5, 10, 20, 30 μg/ml). (**B**) Viability of LO2 cells was determined by the CCK8 assay with 10 μg/ml LPS for 0, 6, 12, and 24 h. (**C**) Flow cytometry of apoptotic LO2 cells between the normal control and LPS-treated (10 μg/ml) groups. (**D**) The protein expressions of Beclin-1 and LC3 in LO2 cells between the normal control and LPS (10 μg/ml) groups for different times determined by western blot. Three independent experiments were performed. * indicates $P<0.05$; ** indicates $P<0.01$

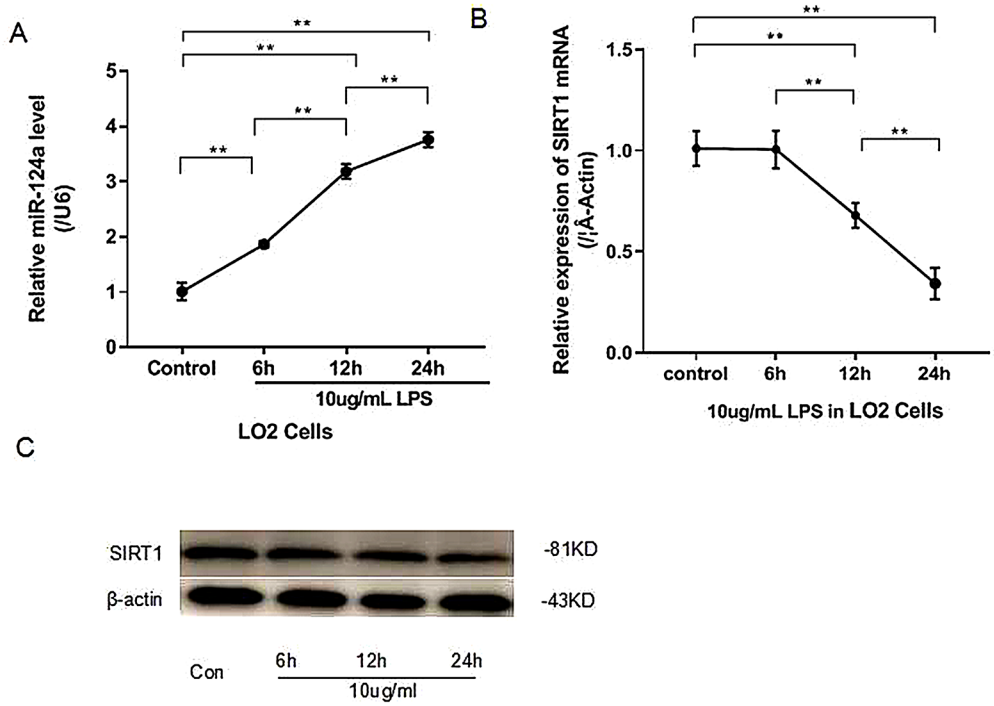

Fig. 4 LPS-mediated downregulation of SIRT1 and upregulation of miR-124a in LO2 cells. The relative mRNA expressions of SIRT1 (**A**) and miR-124a (**B**) between the normal control and LPS (10 μg/ml) groups for 24 h determined by qRT-PCR. (**C**) The SIRT1 protein expressions between the normal control and LPS (10 μg/ml) groups for different times determined by western blot. Three independent experiments were performed. * indicates $P<0.05$; ** indicates $P<0.01$

group (Fig. 5A and B). Further, treatment with miR-124a inhibitors resulted in the notable upregulation of LC3, Beclin-1, and SIRT1, reflecting enhanced autophagy. The SIRT1 mRNA levels were also elevated by miR-124a inhibitors, while the miR-124a mimic caused the opposite effect (Fig. 5C). Those results indicate that miR-124a hampers autophagy via SIRT1. Also, SRT1720 (SIRT1 activator) and EX-527 (SIRT1 inhibitor) were used to prove that SRT1720 upregulated Beclin-1 and LC3, while EX-527 downregulated them (Fig. 5D).

Downregulation of miR-124a inhibits hepatocyte apoptosis Induced by LPS

Cells transfected with the miR-124a mimic showed a significantly higher percentage of apoptosis, while the miR-124a inhibitor significantly reduced apoptosis compared with the control group (Fig. 6A). The BAX protein expression was upregulated, while Bcl-2 protein expression was downregulated in LO2 cells treated with the miR-124a mimic (Fig. 6B). Hence, miR-124a appears to promote apoptosis by enhancing pro-apoptotic factors (BAX) and down-regulation of anti-apoptotic factors (Bcl-2).

Further, to inhibit autophagy in LO2 cells, 3-MA was utilized. Meanwhile, apoptosis was elevated. The effect of 3-MA was partially reversed by co-transfection with the miR-124a inhibitor ($P<0.01$, Fig. 6 C and D). This shows that miR-124a promotes LPS-induced apoptosis and down-regulation thereof counteracts the apoptosis imposed by inhibition of autophagy.

Discussion

In this research, we uncovered the pivotal role that miR-124a plays in ACLF, along with the mechanisms underpinning its influence. Initially, our findings revealed that miR-124 expression was elevated and SIRT1 was down-regulated in ACLF patients and in vitro and was accompanied by autophagy dysfunction and an increase in hepatocyte apoptosis. Additionally, we found the plasma Beclin-1, LC3, SIRT1 and Bcl-2 levels were negatively correlated with miR-124a level, while a positive correlation between miR-124a and BAX expression level. Mechanistically, in vitro downregulation of miR-124a significantly prevented LPS-induced LO2 apoptosis and promoted autophagy via targeting SIRT1.

Considering that chronic diseases are accompanied by persistent hepatocyte apoptosis, which is a causative role in the process of many types of liver injury [12]. Strategies that target hepatic apoptosis have the potential to slow the progression of disease and lower the risk of liver failure.

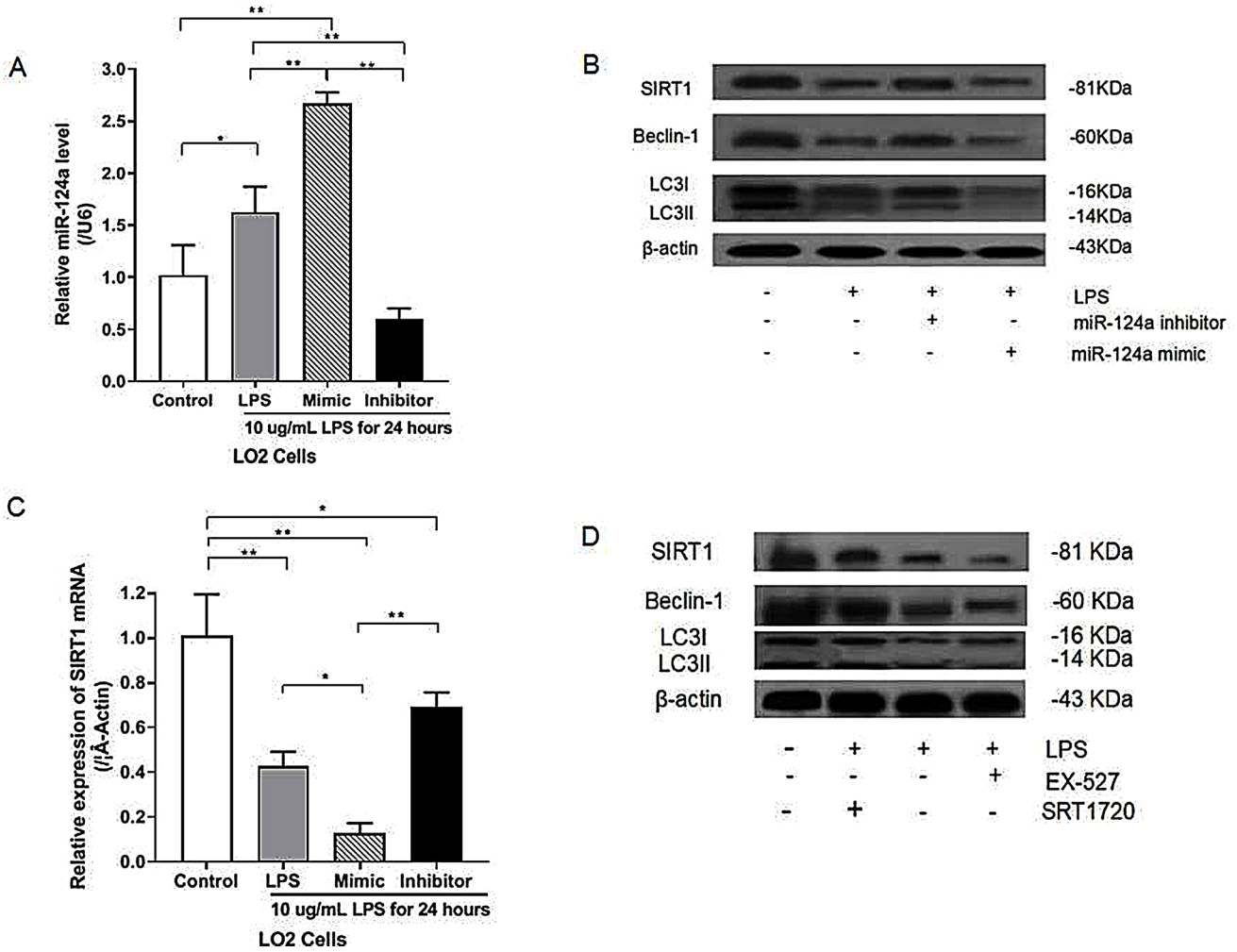

Fig. 5 Upregulated miR-124a further decreased cell autophagy and SIRT1 expression in LO2 cells induced by LPS. (**A**) The relative levels of miR-124a between the normal control, LPS, miR-124a mimic, and miR-124a inhibitor groups determined by qRT-PCR. (**B**) The protein expressions of LC3, Beclin-1, and SIRT1 between the control, LPS, miR-124a mimic, and miR-124a inhibitor groups treated with LPS determined by western blot. (**C**) The relative SIRT1 mRNA expressions between the control, LPS, miR-124a mimic, and miR-124a inhibitor groups treated with LPS determined by qRT-PCR. (**D**) Representative western blot bands showing the protein expressions of LC3 and Beclin-1 after SIRT1 activator (SRT1720), SIRT1 inhibitor (EX-527), and LPS treatments in LO2 cells. * indicates $P<0.05$; ** indicates $P<0.01$

Autophagy serves as a protective cellular process that allows cells to endure adverse conditions and can avert cell death, notably via the inhibition of apoptosis-initiating pathways [12, 24]. Actually, increasing evidence have certified that there was an intricate interplay between autophagy and apoptosis. Moderate levels of autophagy can reduce apoptosis through: (i) the selective degradation of impaired organelles and harmful pathogens, and (ii) the clearance of detrimental debris or abnormal cellular components that may trigger apoptotic responses [4, 12]. Abnormal autophagy (insufficient or excessive) may lead to the execution of apoptotic cell death via Bcl-2 family proteins or autophagic cell death directly [4, 12, 13, 25]. The development of various liver conditions in humans is associated with the disruption of autophagy regulation, including various viral hepatitis, hepatocellular carcinoma, and liver failure [26]. Apoptosis and autophagy are important physiological processes that maintain cell homeostasis and death. Indeed, autophagy levels fluctuate, accommodating shifts in cellular signals and environmental conditions. Research indicates that in a rat model of sepsis induced by CLP, autophagy is temporarily activated in hepatocytes during the early phases. The subsequent reduction in autophagy at later stages may play a role in the progression to liver failure [27]. ACLF is the end-stage liver disease, we observed that the level of autophagy was significantly reduced in patients with ACLF. Ablation of autophagy caused accumulation of damaged organelles. Thus, the autophagic pathway, which was contributed to restore normal cellular function, determined the

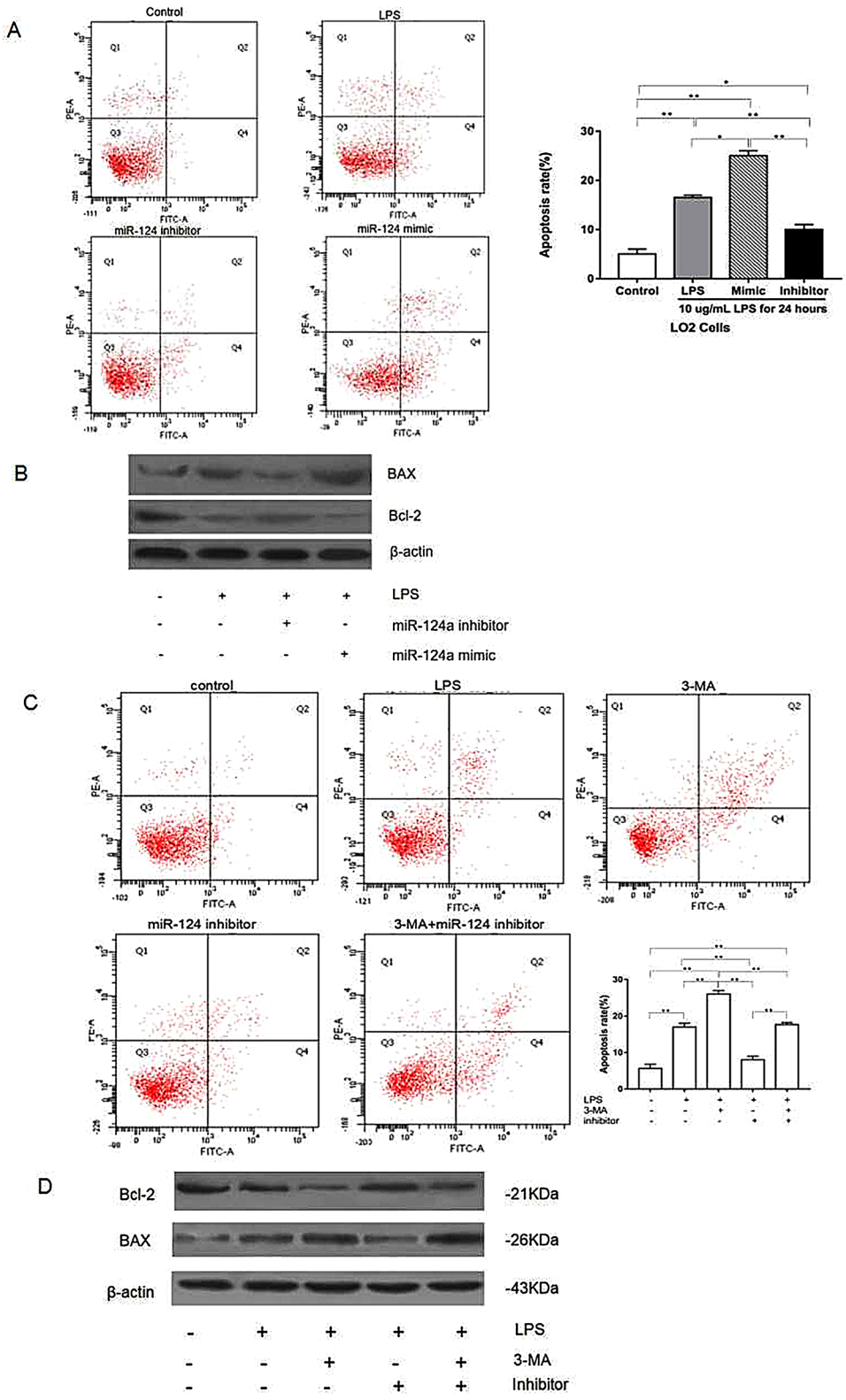

Fig. 6 miR-124a regulated apoptosis by LPS in LO2 cells. (**A**) Subsequent to transfection with miR-124a mimic or inhibitor determined by Annexin V/PI staining followed by flow cytometry to evaluate apoptosis. (**B**) Western blot was used to evaluate apoptosis from the protein expression levels of Bcl-2 and BAX. (**C**) The apoptosis incidence in LO2 cells between the 3-MA, miR-124-3p inhibitor and 3-MA+miR-124 inhibitor groups detected by flow cytometry. (**D**) The protein expressions of Bcl-2 and BAX between the 3-MA, miR-124-3p inhibitor and 3-MA+miR-124 inhibitor groups determined by western blot analysis. * indicates $P<0.05$; ** indicates $P<0.01$

degree of hepatic apoptosis as well as prevent the progression of liver diseases.

Several studies had demonstrated that miRNAs were identified as promising diagnostic or prognostic biomarkers as well as therapeutic targets for various liver diseases involved in liver failure [14–16, 18, 21]. To the best of our knowledge, Individuals suffering from chronic hepatitis B virus infections exhibit a notable elevation in serum miR-124a levels, which are found to be positively associated with the degree of liver necroinflammation [21]. Additionally, prior research indicated that miR-124 exhibited an inverse relationship with BECN1 expression in patients with KRAS-mutant non-small cell lung cancer [28]. MicroRNAs, including miR-124, might play a protective or a disease-promoting role across various illnesses or even at distinct phases of the same condition [6, 14, 16, 18, 20]. Our analysis disclosed an uptick in miR-124a levels among patients with ACLF. Concurrently, our data pointed to a tight link between miR-124a levels in plasma and proteins implicated in autophagy and apoptosis in ACLF patients. To delve deeper into the underlying mechanisms, we developed an in vitro cellular model to explore the impact of LPS on hepatocyte demise. Within the scope of our study, we observed that miR-124a expression rose in LO2 cells subjected to LPS induction, coincident with an increase in hepatocyte apoptosis. Han F et al. have noted that miR-124, by targeting Dhcr24, governs hypoxia and oxidative stress in cardiomyocyte apoptosis [14]. The suppression of miR-124 has been posited as a preventive measure against the apoptotic potential of bladder cancer cells [16]. Zan et al. demonstrated that heightened miR-124 levels led to diminished Bcl-2 expression and a surge in liver cell apoptosis in a murine model of acute liver failure, thereby exacerbating the condition [29]. Notably, our study robustly indicated that the suppression of miR-124a boosted Bcl-2 expression and curbed BAX expression, culminating in decreased hepatocyte apoptosis. In contrast, the overexpression of miR-124a had the opposite effect on BAX and Bcl-2, leading to heightened cell death, which implies a regulatory function of miR-124a in liver injury.

It is recognized that miRNAs carry out their biological roles by attaching to the mRNA of their target genes [17]. A substantial amount of data points to miR-124a's direct interaction with the 3' UTR of SIRT1, thereby inhibiting its deacetylase activity. For instance, Lv et al. [22]. found that upregulation of miR-124 could decrease SIRT1 expression to regulate endometrial cancer growth and metastasis. Similarly, an additional study indicated that an upregulation of miR-124 suppressed SIRT1 expression, consequently enhancing the production of ROS and the phosphorylation of JNK, which triggered apoptosis in $CD133^{+}$ hepatocellular carcinoma cells exposed to cisplatin [23]. SIRT1, a NAD^{+}-dependent deacetylase, by modulating the activities of many transcription regulators to maintain homeostasis including liver injury, is also an important regulator of autophagy [8, 10, 11, 30, 31]. Consistent with the previous findings, our investigation discovered that the mRNA and protein levels of SIRT1 were markedly reduced in liver tissues from ACLF patients and in an LPS-induced hepatocyte death model in vitro. This reduction was negatively associated with the increased levels of miR-124a. Numerous studies have documented that miR-124, known for its tumor-suppressive properties, impedes autophagy by engaging various targets [18]. From the experimental results, we found that miR-124a inhibitor mimicked SIRT1 activator SRT1720 upregulation autophagy, whereas miR-124a mimic mimicked SIRT1 inhibitor EX-527 downregulation autophagy. In additional, Knockdown or overexpression of miR-124a could increase or decrease SIRT1 protein and mRNA expression. The findings highlighted that miR-124a could suppress autophagy by repressing the expression of SIRT1. Notably, it became evident that Bcl-2 family members, including Bcl-2 itself, not only neutralize the effects of pro-apoptotic proteins to reduce apoptosis but also engage with Beclin-1, hindering its binding to the PI3KC3 complex and thus obstructing autophagy [5, 25]. Additionally, the application of 3-MA, an inhibitor of autophagy, resulted in diminished levels of the anti-apoptotic Bcl-2 protein and a corresponding increase in the pro-apoptotic BAX protein levels. This suggests that the promotion of apoptosis was associated with the suppression of autophagy. In comparison to the group that received 3-MA only, the apoptosis rate among LO2 cells was decreased when treated with both 3-MA and an inhibitor of miR-124a. This suggests that inhibiting miR-124a may mitigate the pro-apoptotic impact caused by the autophagy inhibitor 3-MA on LO2 cells. All above experimental results may give us a hint that miR-124a reduced autophagy and promoted apoptosis by negatively modulating the downstream target gene SIRT1. As reported, miR-124a is predicted have many potential targets, several predicted miR-124a targets, including PIM, BACE1, MALAT1, STAT3, PDCD6, Casp-3 and AMPK/mTOR, which affect cellular autophagy, proliferation, cell cycle, apoptosis, and cell growth control [18]. It is conceivable that miR-124a modulates autophagy and apoptosis in ACLF through the interplay of several signaling pathways, and additional research is warranted to uncover these mechanisms. Here we found that SIRT1 may be one of key molecule targets of miR-124a that contribute to hepatocyte apoptosis and autophagy during LPS-induced LO2 cells.

The study had several limitations. (i) Limited clinical sample size; (ii) lack of in vivo study; (ii) lack of luciferase assay, which could potentially diminish the persuasiveness of the evidence regarding liver failure by miR-124a. Further

studies with luciferase assay and reliable laboratory animals should be conducted in the future to establish a more reliable observation.

Conclusion

In conclusion, our study revealed an inverse correlation between miR-124a levels and SIRT1 expression in clinical samples and in vitro studies. The suppression of miR-124a had a significant protective impact on hepatocytes by inhibiting apoptosis and enhancing autophagy through the upregulation of SIRT1, a confirmed target of miR-124a. Further exploration into the miR-124a/SIRT1 axis may uncover its potential as a biomarker and a promising therapeutic target for ACLF treatment.

Declarations of originality

The research presented here has not been previously published. Its dissemination has been approved by all authors and tacitly permitted by the relevant research institutions.

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Author contributions Hongrui Xu carried out study concepts & design, and manuscript editing; Xin Wang contributed to clinical studies, data & statistical analysis; Yadong Wang, Chuan Shen and Luyuan Ma contributed to definition of intellectual content and data acquisition; Caiyan Zhao were the guarantor of integrity of the entire study, helped to literature research and manuscript review.

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Data availability The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Ethical approval All procedures performed in this study involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Ethics Committee of the Third Hospital of Hebei Medical University. Written informed consent was obtained from all participants.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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