

## MicroRNA-181a-5p alleviates acute liver failure in mice by inhibiting HMGB1

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### ABSTRACT

MicroRNAs (miRNAs) control liver diseases, but the role of microRNA-181a-5p in acute liver failure (ALF) is unclear. In this study, the ALF model was generated by injection of D-galactosamine (D-GalN) and lipopolysaccharide (LPS). The levels of miRNAs were assessed by microarray and qRT-PCR. The expression of caspase 3 was detected as the marker of cell apoptosis in ALF by immunohistochemistry and western blot. The targeting of microRNA-181a-5p on the high mobility group box 1 (HMGB1) was verified by dual luciferase assay. The impact of microRNA-181a-5p and HMGB1 was explored by flow cytometry. Results showed that microRNA-181a-5p was significantly down-regulated by D-GalN/LPS in vivo and in vitro, while the level of HMGB1 was up-regulated after the challenge. Furthermore, microRNA-181a-5p overexpression attenuated cell apoptosis in D-GalN/TNF-treated BNLCL2 cells. MicroRNA-181a-5p could directly target HMGB1 mRNA and repress its expressions, in further HMGB1 is involved in microRNA-181a-5p effect on cell apoptosis of ALF. In conclusion, these findings demonstrate that microRNA-181a-5p regulates hepatocyte apoptosis via HMGB1 in the development of ALF, which may provide potential therapeutic targets for ALF. However, the precise underlying mechanism that connects microRNA-181a-5p and HMGB1 remains to be explored.

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### Introduction

ALF is a disease in which liver cells are suddenly damaged and may rapidly lead to multi-organ failure over days or weeks (1). Acetaminophen overdose, autoimmune hepatitis, hepatic ischemia, and herbal and dietary supplements, especially drug-induced injury and viral hepatitis are the main causes of drug-induced injury (2). With the development of society, the prevalence of liver disease is increasing, but treatment options for its complications are limited (3). Liver transplantation is the primary treatment for ALF, but insufficient donor livers increase lethality (4). The development of ALF is accompanied by the immune response, which leads to apoptosis and reduced proliferation and has attracted strong interest in recent years, but the exact mechanism remains to be investigated (5).

MiRNAs target the 3'UTR of mRNAs to control their expressions (6). MicroRNA could change cellular function and gene expression in health and disease by sorting sequences that determine their secretion in sEVs or cellular retention (7). Increasing evidence indicates that miRNAs regulate several biological processes (8). MicroRNA-181a-5p was found to play a significant role in liver damage or non-alcoholic fatty liver disease (9, 10). However, the regulatory mechanism of microRNA-181a-5p in ALF remains to be elucidated, and whether there is a link between microRNA-181a-5p and hepatocyte apoptosis is unclear. Therefore, it is desirable to exploit the roles of microRNA-181a-5p. Here, we demonstrate that microR-

NA-181a-5p regulates hepatocyte apoptosis in a murine ALF model.

### Materials and Methods

#### Experimental ALF model

The experiments were approved by the laboratory animal care committee and followed the guidelines. 6-8 weeks old male BALB/c mice were kept under standard conditions with food and water. 600 mg/kg D-galactosamine (Sigma, USA) and 10 µg/kg LPS were intraperitoneally injected into the experimental mice (n= 30) and the mice were sacrificed at different times (0, 3, 6, 9, and 12 h; 6 mice per time). Meanwhile, saline was applied to the control group (n= 30) and mice were also sacrificed at the same time points. Liver tissue samples (100 mg mixture of liver tissues from 6 mice per sample) from the GalN/LPS treatment group or control group (3 samples per group; 0, 6, and 12 h treatment, respectively) were performed miRNA array analysis; the remaining liver tissues were used for qRT-PCR, western blot, histopathology, and immunohistochemistry.

#### MiRNA microarray

MiRanda and TargetScan databases predicted the targets of microRNA-181a-5p. RNA was extracted using Isolation Kit (Solarbio, China). Microarray (Agilent, USA) was used to screen RNA samples. The labeling and hybridization were executed with Biochip (Shanghai, China).

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The microarray slides were scanned by Agilent Scan Control, and the images were analyzed by Agilent Feature Extraction. The data were processed under GeneSpring GX (Agilent, USA).

### Cell cultures and transfection

HEK293T and BNLCL2 cells were maintained in DMEM (Thermo Fisher, USA) with 10% FBS (Gibco, USA) and antibiotics at 37° C with 5% CO<sub>2</sub>. To induce hepatocyte apoptosis, 1 mg/ml D-GalN (Solarbio, China) and 100 ng/ml TNF- $\alpha$  (Solarbio, China) were applied. 20 nmol/L of microRNA-181a-5p mimic, microRNA-181a-5p inhibitor or nonrelative control (NC) duplex (GenePharma, China) or HMGB1 siRNAs or NC siRNA (Ribobio, China) were transfected into the cells with lipofectamine 3000 (Thermo Fisher, USA)

### RT-qPCR

RNA was extracted with Trizol (Solarbio, China) and reverse transcribed into cDNA. The RNA level was measured using TaqMan MicroRNA Kit (AB, USA) and the 7500 PCR system (AB, USA). Relative quantification analysis was conducted by the 2<sup>- $\Delta\Delta$ CT</sup> method.

### Cell apoptosis assay

Transfected cells were resuspended and incubated with FITC-Annexin V (Promega, USA) at 4° C in the dark. After staining, the cells were incubated with PI at 4° C and then analyzed by Cell Lab Quanta (Beckman-coulter, USA) flow cytometry.

### Immunohistochemistry

Liver tissues were deparaffinized and rehydrated. Anti-caspase-3 antibodies were applied at 4° C overnight, followed by HRP-conjugated goat anti-rabbit antibodies (Abcam, USA). Finally, DAB and 20% hematoxylin were applied.

### Western blot

Proteins were extracted and measured with the BCA method. After separating using 10% polyacrylamide gels and transferring them onto PVDF membranes (Millipore, USA), the primary and secondary antibodies (Abcam, USA) were applied and the bands were scanned.

### Luciferase reporter assay

The 3'-UTR fragment of HMGB1 was amplified. The product was cloned into a vector (pGL3; Promega, USA). A plasmid containing mutations was generated using a MutanBEST Kit (Takara, Japan). HEK293T cells were co-transfected and analyzed using the dual-luciferase reporter kit (Promega, USA).

### Statistical analysis

Data were presented as the means  $\pm$  SD. SPSS 19.0 was executed. A one-way ANOVA test, least significant difference (LSD) test, Chi-square test, and Student's test were used for analysis.  $p < 0.05$  indicated significance.

## Results

### Deregulated microRNA-181a-5p in the ALF model

In the challenged mice, the mortality rate was 60% and 80% at 6 h and 12 h, respectively, while no deaths were de-

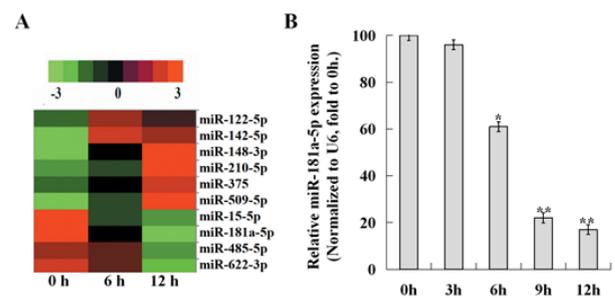
tected in the control group. Some miRNAs were found to be deregulated after D-GalN/LPS challenge. The upregulated (miR-122-5p, miR-142-5p, miR-148-3p, miR-210-5p, miR-375 and miR-509-5p) and downregulated miRNAs (miR-15-5p, miR-181a-5p, miR-483-5p and miR-622-3p) are shown in Fig 1A. MicroRNA-181a-5p was the most significant and it was confirmed that microRNA-181a-5p expression was gradually down-regulated from 6 to 12 hours after challenge in the ALF model (Fig. 1B).

### Increased hepatocyte apoptosis in the ALF model

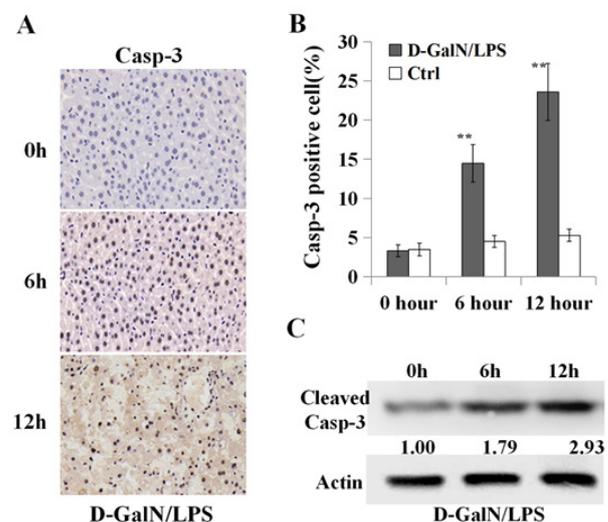
The hepatocyte apoptosis was analyzed. The caspase-3-positive cells were found in the liver of experimental mice at 6 h post-challenge, an increase in positive cells was observed at 12 h post-challenge, while there were no changes of positive cells in the saline-treated group (Fig. 2A and 2B). To confirm these results, a western blot of caspase-3 was performed. As expected, the challenged mice showed increased caspase-3 expression at both the 6 h and 12 h time points compared with saline-treated mice (Fig. 2C).

### MicroRNA-181a-5p overexpression attenuated cell apoptosis

To investigate whether microRNA-181a-5p regulated hepatocyte apoptosis in vitro, a cell apoptosis assay



**Figure 1.** MicroRNA-181a-5p was down-regulated in ALF. A. The differentially expressed miRNAs were identified. B. qRT-PCR analysis of microRNA-181a-5p expression at hours 0, 3, 6, 9 and 12h. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 2.** Cell apoptosis was detected. A. Apoptosis was observed by caspase-3 immunohistochemistry staining in liver tissues. B. Quantification of caspase-3 positive cells in panel A. C. Western blot of cleaved caspase-3 in liver tissues of D-GalN/LPS or saline treatment. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

was performed. The expression of microRNA-181a-5p was promoted in cells transfected with microRNA-181a-5p mimic but dropped in cells transfected with microRNA-181a-5p inhibitor (Fig. 3A). As the control, the apoptotic rate was around 3.5 ~ 4.2% and no significance was found in BNLCL2 cells without D-GalN/TNF- $\alpha$  (Fig. 3B). However, the apoptotic rate was 11.34% in BNLCL2 cells 6 h after induction, while microRNA-181a-5p mimic caused a decreased apoptotic rate to 8.96% after induction (Fig. 3B). No change detected in the apoptotic rate in NC duplex transfected cells or non-transfected cells. Consistent with these results, transfection of microRNA-181a-5p mimic repressed the expression of caspase-3, but transfection of microRNA-181a-5p inhibitor enhanced it (Fig. 3C) in BNLCL2 cells.

### HMGB1 targeted microRNA-181a-5p

To confirm the relationship between microRNA-181a-5p and HMGB1 (Fig. 4A), the luciferase report assay was used. The pGL3-HMGB1-3'UTR (wt/mut) luciferase report plasmids and microRNA-181a-5p mimic, microRNA-181a-5p inhibitor or NC were transfected into HEK293T cells. MicroRNA-181a-5p mimics could repress the luciferase activity of the reporter carrying wild-type HMGB1 3'UTR sequence (Fig. 4B). In addition, microRNA-181a-5p mimic also weakened HMGB1 protein expression (Fig 4C). Moreover, hepatic HMGB1 protein levels were up-regulated in the ALF model from 3 h to 12 h (Fig. 4D). These findings suggested that HMGB1 is the target of microRNA-181a-5p.

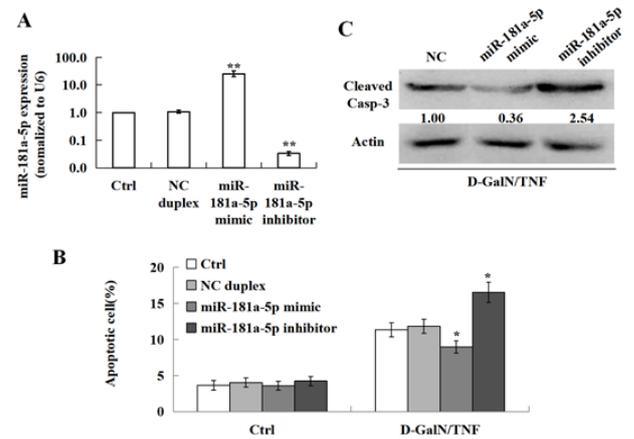
### MicroRNA-181a-5p induced apoptosis by modifying HMGB1 expression

To explore whether HMGB1 is involved in microRNA-181a-5p's influence on cell apoptosis in ALF, the effect of HMGB1 knockdown was verified. Firstly, transfection with HMGB1-specific siRNA could repress the expression of HMGB1 (Fig 5A). Knockdown of endogenous HMGB1 (Fig 5B) decreased cell apoptosis, similar to the effect of microRNA-181a-5p. Furthermore, HMGB1 siRNA, but not NC siRNA partly rescued cell apoptosis enhanced by microRNA-181a-5p knockdown (Fig 5B). Thus, it is possible that microRNA-181a-5p can modulate cell apoptosis in ALF by regulating HMGB1.

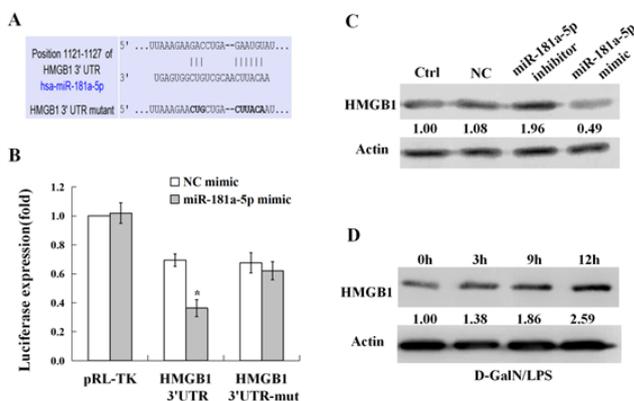
### Discussion

Key pathologic traits of ALF are necrosis and apoptosis, but the exact underlying mechanism remains to be elucidated (11). Caspases are mediators of programmed cell death. Caspase-3 is activated frequently and catalyzes the specific lysis of many crucial cellular proteins (12). Cytotoxic cell apoptosis is accompanied by an upregulation of caspase-3 positive cells in the liver of ALF. In the past decade, miRNAs could be potential biomarkers for different diseases, including ALF (13, 14). For instance, miR-33a, miR-203b, and miR-361-3p contribute to hepatocellular carcinoma pathogenesis and potentially be used as noninvasive biomarkers for cancer therapy (15). In addition, there are several miRNAs reported to be involved in ALF, such as miR-24 (16), miR-1224 (17), miR-210 (18), miR-125b-5p (19), etc.

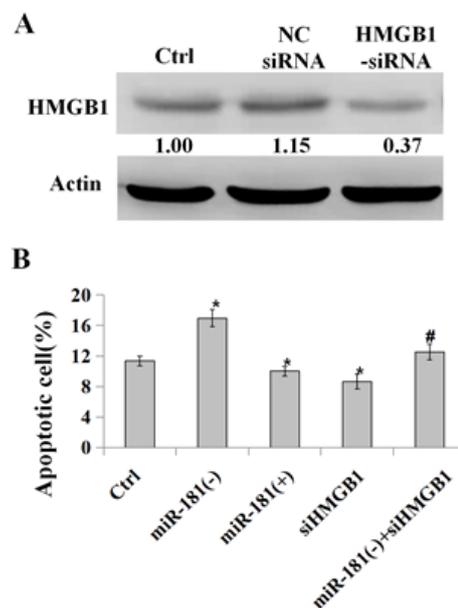
Impressively, MicroRNA-181a-5p controls many biological processes including apoptosis. Previously, studies



**Figure 3. MicroRNA-181a-5p attenuated cell apoptosis in ALF.** A. qRT-PCR analysis of microRNA-181a-5p level. B. Cell apoptosis assay in transfected cells was performed. C. western blot of cleaved caspase-3 in transfected cells was performed. \* $P < 0.05$ .



**Figure 4. MicroRNA-181a-5p sponged HMGB1.** A. Predicted binding site between 3'UTR of HMGB1 and microRNA-181a-5p. B. Dual luciferase assay verified the prediction. C. Western blot of HMGB1 expression in cells. D. Western blot of HMGB1 expressions in liver tissues. \* $p < 0.05$ .



**Figure 5. MicroRNA-181a-5p sponged HMGB1 mediating cell apoptosis.** A. Western blot of HMGB1 expression. B. Quantification of the cell apoptosis assay. \* $p < 0.05$ , \*\* $p < 0.01$ .

reported the role of microRNA-181a during immunity activation (20), inflammation (21), and cancer progression (22). In recent years, theoretical and experimental investigations of microRNA-181a-5p increased tremendously. Zhang M, et al. reported that microRNA-181a-5p targeting HMGB1 suppresses the NF- $\kappa$ B pathway to attenuate inflammatory response (23). A study reported that microRNA-181a-5p regulated DC responses to HMGB1 and may be a potential treatment for immune dysfunction in the setting of sepsis (24). Also, microRNA-181a-5p overexpression reduced asthma progression via repressing the signaling way of HMGB1/RAGE inflammation by inhibiting HMGB1 (25). What's more, it has been highlighted that microRNA-181a-5p, which targeted HMGB1, induced M1 polarization of macrophages, potentially be the target for treating acute pancreatitis (26). Guo L, et al. found that microRNA-181a-5p/HMGB1 regulated pyroptosis-mediated cell death and alleviated Spinal cord ischemia-reperfusion injury by activating caspase1 (27). Moreover, microRNA-181a-5p alleviated neuron diseases by sponging HMGB1 (28). Altogether, these data suggest that HMGB1 could be regulated by the microRNA-181 family. However, the precise underlying mechanism that connects microRNA-181a-5p and HMGB1 during ALF remains to be explored. Here, we demonstrated that microRNA-181a-5p regulated hepatocyte apoptosis by targeting HMGB1 during ALF.

HMGB1 facilitates DNA replication, transcription, recombination and repair (29). Dimerized HMGB1 reduced DNA hydroxyl free radicals damage and prevented cell death (30). In the cytoplasm, HMGB1 can promote autophagy by binding to the BECN1 protein (31). Extracellular HMGB1 contributes to cytokine production and induces immune cell recruitment via the activation of various receptors (32). As a prominent autophagic regulator (33), HMGB1-mediated autophagy showed the effects of inhibition of apoptosis and promoting drug resistance in various tumor types (34). Recently, studies reported several miRNAs played roles in different diseases by targeting HMGB1. HMGB1 could be regulated by miR-128-1 in high-fat-diet-induced obesity (35), miR-200b-3p in Maturity-onset diabetes of the young (36), miR-142-3p in non-small-cell lung Lung Adenocarcinoma (37), miR-493-5p and miR-200 in hepatocellular carcinoma (38, 39), miR-34a in cervical cancer (40), miR-218 in prostate cancer (41), et al. However, the miRNA regulation of HMGB1 in ALF has yet to be identified.

In this study, we speculate that microRNA-181a-5p overexpression reduces HMGB1 expression, which downregulates downstream caspase-3 expression to limit hepatocyte apoptosis. In light of our data demonstrating that microRNA-181a-5p regulates hepatocyte apoptosis by HMGB1 during ALF. Despite these considerable advantages, the limitation of the study must be noticed. The precise underlying mechanism that connects microRNA-181a-5p and HMGB1 remains to be explored. The exact mechanism is subject to further research and discussion. To this end, the upstream regions of microRNA-181a-5p are currently being investigated.

In conclusion, our study revealed that microRNA-181a-5p possesses the ability to attenuate hepatocyte injury and regulate hepatocyte apoptosis by targeting HMGB1. We illustrated that microRNA-181a-5p overexpression reduces HMGB1 expression, correspondingly downre-

gulates downstream genes, and caspase-3, consequently attenuating hepatocyte apoptosis. MicroRNA-181a-5p is a potential regulator and biomarker of ALF. We provide new insights into the treatment of ALF and its potential mechanism. Targeting HMGB1 by regulating microRNA-181a-5p can be a novel therapy for ALF and its complications. However, the exact underlying mechanism that connects microRNA-181a-5p and HMGB1 remains elusive. In the future, we will explore the potential precise mechanism of microRNA-181a-5p and HMGB-5p linkage in ALF.

### Ethical approval

All animal care and experimental procedures complied with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiment was approved by the Institutional Animal Care and Utilization Committee of Linyi People's Hospital.

### Authors contribution

Qianwen Zhang conceived the idea and designed the research study. Hao Xu and Zengxi Xue performed most experiments and analyzed the data. Yuqiao Zeng wrote the manuscript. Li Wang and Qingyun Sun repeated some experiments. Yiyu He provided help and advice on animal experiments and analyzed the data. Likun Wang provided advice on the study and manuscript.

### Consent to publish

All authors gave final approval of the version to be published.

### Competing interests

The authors report no conflict of interest.

### Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Not applicable.

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