Abstract. Acute liver failure (ALF) is a fatal liver disease characterized by severe hepatocyte destruction. MicroRNAs (miRNAs/miRs) have been reported to serve a key role in a number of liver diseases. Therefore, the aim of the present study was to investigate the role and underlying mechanism of miR-214 in ALF. ALF murine and hepatocyte models were established using D-galactosamine (D-Galn) and lipopolysaccharide (LPS) or D-Galn + tumor necrosis factor (TNF)-α, respectively. The expression levels of miR-214 and Bax were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and/or western blotting. Furthermore, an automatic biochemical analyzer was used to measure the levels of aspartate aminotransferase (AST) or alanine aminotransferase (ALT). The levels of TNF-α and interleukin (IL)-6 were detected by ELISA and RT-qPCR. In addition, TUNEL staining and flow cytometry were used to analyze cell apoptosis, and the protein expression of caspase-3 was determined by western blotting. It was identified that the levels of AST and ALT were increased and that hepatocyte apoptosis was enhanced in the D-Galn/LPS-stimulated group compared with the control. Furthermore, higher expression of caspase-3 was observed in the D-Galn/LPS-stimulated group. In addition, it was demonstrated that miR-214 was downregulated, while Bax was upregulated in D-Galn/LPS-stimulated mice and D-Galn/TNF-α-stimulated BNCL2 cells. Moreover, in D-Galn/TNF-α-stimulated BNCL2 cells, miR-214 overexpression suppressed apoptosis and decreased TNF-α and IL-6 levels, and these effects were reversed by the Bax plasmid. It was also identified that overexpression of miR-214 significantly decreased Bax mRNA and protein expression levels in vitro. Collectively, the present results suggested that miR-214 inhibited hepatocyte apoptosis during ALF development via targeting Bax, thus indicating that miR-214 may be a potential target for ALF treatment.

Introduction

Acute liver failure (ALF) is a rapidly developing disease, with a rapid onset of symptoms, that is associated with multi-organ failure (1). The main causative factors of ALF include drug toxicity and hepatitis virus infection (2). D-galactosamine (D-Galn) and lipopolysaccharide (LPS) are common biochemical reagents that may be used to establish fulminant hepatic failure (FHF) injury models, which effectively simulate the FHF clinical state (3). During ALF development, one of the key pathological traits is the associated immoderate immune cascade response, which leads to extensive liver cell apoptosis and defective liver cell proliferation (4). However, little is known regarding the mechanism of action of microRNAs (miRNAs) in the process of ALF (5).

miRNAs are a class of endogenous non-coding RNAs, measuring 19-22 nucleotides in length, that regulate the expression of target genes by interacting with the 3′-untranslated (UTR) regions of these genes at the post-transcriptional level (6). Furthermore, miRNAs have been identified as potential biomarkers in the pathological processes of several life-threatening diseases, including ALF (7). Abnormal regulation of miRNAs has also been observed in various liver diseases, and miRNAs serve a key role in regulating hepatocyte proliferation (8) and liver development (9,10). For example, miR-122 is one of the most common miRNAs in the liver, and is involved in appropriate proliferation and differentiation of liver cells (11). In addition, miR-122 has been implicated in viral hepatitis and liver disease (12). Moreover, it has been reported that miR-125b-5p could inhibit ALF (13). It has also been suggested that miR-214 exerts anti-fibrotic effects in chemically induced liver fibrosis and cirrhosis (14). However, the role of miR-214 in human ALF remains unknown.

Therefore, the aims of the present study were to investigate the role of miR-214 in ALF and to elucidate its mechanism of action.
Materials and methods

Experimental animals and study design. A total of 30 male BALB/c mice (age, 6-8 weeks; weight, 20-22 g) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., and were housed in a standard animal housing facility (temperature, 22-24°C; humidity, 60-65%) with ad libitum access to food and water under a 12 h light/12 h dark cycle. The mice were randomly divided into two groups (control and ALF model groups; n=15/group). To establish the mouse model of ALF, the mice were administered D-GalN [800 mg/kg body weight intraperitoneal (i.p.); Sigma-Aldrich; Merck KGaA] and LPS (10 µg/kg body weight, i.p.; Sigma-Aldrich; Merck KGaA) as described previously (15). Mice in the control group were treated with 500 µl saline by i.p. injection. Mice were anesthetized with pentobarbital (50 mg/kg) by i.p. injection and sacrificed by cervical dislocation to collect blood samples (1 ml) at 0, 1, 3, 5, 7 and 9 h after D-GalN/LPS treatment for aspartate aminotransferase (AST) or alanine aminotransferase (ALT) detection. Animal death was defined as the lack of heartbeat or respiration. The blood (1 ml) of mice at 7 h after D-GalN/LPS treatment was collected for interleukin (IL)-6 and tumor necrosis factor (TNF)-α detection.

All animal care and experimental protocols were performed strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and the Animal Ethics Committee of The First Affiliated Hospital of Suzhou University. The present study was approved by the Animal Ethics Committee of The First Affiliated Hospital of Suzhou University. Moreover, there was no mouse mortality during the aforementioned experimental procedures. The experimental end-point was when mice lost >15% of their body weight.

Cell culture and treatment. Normal murine embryonic liver cells (BNLCL2) were provided by Wuhan Procell Life Technology Co., Ltd. (https://www.procell.com.cn/view/537. html) and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 4 mM glutamate and 1% penicillin/streptomycin (Gibco/Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified chamber with 5% CO₂.

BNLCL2 cells were treated with 1 mg/ml D-GalN (Sigma-Aldrich; Merck KGaA) and 100 ng/ml TNF-α (Sigma-Aldrich; Merck KGaA) at 37°C for 6 h to induce the hepatocyte injury model in vitro.

For miR-214 mimic treatment, BNLCL2 cells were transfected with 100 nM mimic control (sense, 5'-UUUGUA CUACACAAAAGUACUG-3' and anti-sense, 5'-CAUUGA UUUUGUGAUCAAA-3'); Guangzhou RiboBio Co., Ltd.), 100 nM miR-214 mimic (sense, 5'-ACAGCGAGC AGACAGCGAGCAGU-3' and anti-sense, 5'-ACUGCCGUG CUGUGCCGUGUGU-3'; Guangzhou RiboBio Co., Ltd.) or 100 nM miR-214 mimic + 1 µg Bax CRISPR activation plasmid (cat no. sc-49292-ACT; Santa Cruz Biotechnology, Inc.) for 24 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, cells were treated with D-GalN (1 mg/ml) and TNF-α (100 ng/ml) at 37°C for 36 h and used for further analysis.

Transfection of miR-214 mimic in cells. miRNA mimic is small double-stranded RNA oligonucleotide, which can simulate endogenous mature miRNA molecules (16). The synthesized miR-214 mimic was purchased from Guangzhou RiboBio Co., Ltd. BNLCL2 cells were transfected with miR-214 mimic, mimic control, Bax plasmid, control-plasmid or miR-214 mimic + Bax plasmid using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. Then, 24 h after cell transfection, the efficiency of transfection was analyzed using reverse transcription-quantitative PCR (RT-qPCR).

Luciferase reporter assay. miRNA.org software (http://www. mirorna.org/microrna/getMirnaForm.do; August 2010 Release) was used to predict the potential target of miR-214. To assess the association between miR-214 and Bax, wild-type (WT) and mutant (MUT) 3'-UTR of Bax containing the miR-214 binding sites, were amplified by RT-PCR using a Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics), incubating for 5 min at 25°C followed by 60 min at 42°C, from total RNA preparations extracted from BNLCL2 cells and cloned into the psiCHECKTM-2 vector (Promega Corporation). The following primer sequences were used: Bax forward, 5'-GGACGAACACT GGACAGTAACATTG-3' and reverse, 5'-GCAAAGTAGAAAA AGGGCGCACAC-3'. Then, 100 ng psiCHECK-2 luciferase reporter plasmids containing WT and MUT 3'-UTR of Bax were co-transfected into BNLCL2 cells with miR-214 mimic (100 nM) or mimic control (100 nM) for 48 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, a Dual Luciferase Assay system (Promega Corporation) was used to detect luciferase activity in the transfected cells. Renilla luciferase activity was used as the control.

ALT and AST detection assay. The levels of AST and ALT were detected in the blood of mice to assess liver injury. Blood (0.1 ml) was collected from each mouse to analyze the serum levels of ALT and AST. The samples were centrifuged at 8,000 x g for 8 min at 4°C and an automatic biochemical analyzer (Hitachi Ltd.) was used to determine the serum ALT and AST levels according to the manufacturer's protocol.

ELISA. Serum levels of TNF-α (Mouse TNF-α ELISA kit; cat no. PT512) and IL-6 (Mouse IL-6 ELISA kit; cat no. PI326) in D-GalN/LPS-treated mice, and those in the supernatant (centrifugation at 500 x g at 4°C for 5 min) of BNLCL2 cells were determined by ELISA kits (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

TUNEL staining. TUNEL staining was performed to determine cellular apoptosis post-D-GalN/LPS challenge using the in situ cell death detection kit (cat no. 11684817910; Roche Diagnostics) following the manufacturer's instructions. Cells in each group were fixed with 4% paraformaldehyde at room temperature for 15 min, and dewaxed and hydrated liver tissue sections were permeabilized with 0.1% Triton X-100 solution for 15 min. Then, 50 µl TUNEL reaction mixture (Roche Diagnostics) containing terminal deoxynucleotidyl transferase and fluorescein-dUTP was added into the sample. Subsequently, the cells were incubated at 37°C in the dark for 60 min, washed three times with PBS solution for 5 min.
D-GalN/LPS induces acute liver injury in mice. Liver function and/or the extent of damage in the liver tissues were evaluated by serum ALT and AST levels. Mice were sacrificed at various time points (0, 1, 3, 5, 7 and 9 h) after D-GalN/LPS challenge, and blood was collected for serum ALT and AST analysis. It was identified that serum ALT and AST levels gradually increased over the 9 h post-D-GalN/LPS stimulation, peaking at 7 h, compared with the control group, suggesting the presence of liver damage in the mice (Fig. 1a and B). The results also suggested that the maximum level of liver damage was reached at 7 h after D-GalN/LPS stimulation, thus mice at 7 h after D-GalN/LPS stimulation was selected in subsequent experimentations.

IL-6 and TNF-α are involved in hepatocyte apoptosis and regeneration (18,19). Therefore, the levels of IL-6 and TNF-α in the serum of mice were measured after D-GalN/LPS treatment. It was demonstrated that the levels of IL-6 and TNF-α were significantly increased at 7 h post-D-GalN/LPS challenge compared with saline-treated group (Fig. 1C and D).

D-GalN/LPS stimulates hepatocyte apoptosis in mice. Apoptosis of hepatocytes was assessed by TUNEL staining, and it was identified that the percentage of apoptotic cells was significantly increased at 7 h post-D-GalN/LPS challenge compared with the saline-treated group (Fig. 2A). Furthermore, the expression of the apoptotic-associated protein caspase-3 was analyzed, and D-GalN/LPS-challenged mice exhibited increased caspase-3 protein expression at 7 h post D-GalN/LPS challenge compared with saline-treated mice (Fig. 2B).

miR-214 is downregulated in D-GalN/LPS-stimulated mice. The RT-qPCR results demonstrated that the mRNA expression
of miR-214 was significantly downregulated in the liver tissue of D-GalN/LPS-stimulated mice compared with the saline control group (Fig. 3A).

Bax is a target gene of miR-214 and is upregulated in D-GalN/LPS-stimulated mice. miRNA.org software predicted the binding sites between Bax and miR-214 (Fig. 3B), and a
dual luciferase reporter assay was conducted to examine the identified miR-214 binding sites in the 3'-UTR of Bax. The results indicated that treatment with the mir-214 mimic decreased the relative luciferase activity of Bax-WT, but had no effect on Bax-MUT, compared with the mimic control group (Fig. 3c). Therefore, it was speculated that Bax may be a target gene of mir-214. In addition, Bax mRNA (Fig. 3d) and protein (Fig. 3E) expression levels were significantly increased in the liver tissue of mice at 7 h post d-Galn/LPS stimulation compared with the saline control group.

**miR-214 is downregulated and Bax is upregulated in D-GalN/TNF-α-stimulated hepatocytes.** Subsequent experiments were conducted in an in vitro model of BNLCL2 cells stimulated by D-GalN and TNF-α. The expression of miR-214 was first detected in BNLCL2 cells treated with (model) or without (control) D-GalN/TNF-α. The results indicated that, compared with the control group, miR-214 expression was significantly decreased in D-GalN/TNF-α-treated BNLCL2 cells (Fig. 4A). In addition, compared with the control group, Bax was significantly increased in D-GalN/TNF-α-treated BNLCL2 cells at both the mRNA (Fig. 4B) and protein expression levels (Fig. 4C).

**miR-214 mimic inhibit cell apoptosis and inflammation in D-GalN/TNF-α-stimulated hepatocytes.** To investigate the regulatory role of miR-214 in D-GalN/TNF-α-stimulated hepatocytes, BNLCL2 cells were transfected with mimic control, miR-214 mimic or miR-214 mimic + Bax plasmid for 24 h; subsequently, cells were treated with D-GalN (1 mg/ml) and TNF-α (100 ng/ml) for 36 h. Transfection efficiencies were detected by RT-qPCR, and it was identified that miR-214 overexpression resulted in the downregulation of the mRNA and protein expression levels of Bax in BNLCL2 cells, and this downregulation was reversed by Bax plasmid transfection (Fig. 5c and d).

Furthermore, flow cytometry results demonstrated that D-GalN/TNF-α treatment significantly enhanced BNLCL2 cell apoptosis compared with the control group. Moreover, compared with the D-GalN/TNF-α treatment alone group, the results indicated that the miR-214 mimic significantly decreased BNLCL2 cell apoptosis, which was reversed by Bax plasmid transfection (Fig. 6a and B).
In concordance with the *in vivo* results, the mRNA and protein expression levels of TNF-α (Fig. 7A and C) and IL-6 (Fig. 7B and D) in BNLCL2 cells post-D-GalN/TNF-α challenge were significantly increased compared with the control group. Furthermore, the results suggested that miR-214 mimic transfection significantly decreased the mRNA expression and protein levels of Bax.
protein levels of TNF-α and IL-6 in BNLCL2 cells stimulated by D-GalN/TNF-α, and all of these changes were reversed by the Bax plasmid.

Discussion

ALF is a condition associated with high mortality (1), but its underlying pathological mechanism remains largely unknown. Therefore, the development of novel prognostic biomarkers and therapeutic targets for ALF is crucial. miRNAs have been reported to regulate various aspects of hepatic function, including cell proliferation, metabolism and viral infection (20). The present results demonstrated that miR-214 was downregulated and Bax was upregulated in a D-GalN/LPS-induced murine ALF model. In addition, the results indicated that miR-214 ameliorated ALF via the regulation of Bax expression.

D-GalN/LPS has been widely used to induce hepatic damage, accompanied by changes in hepatic apoptosis and necrosis, which are similar to the changes observed in human viral hepatitis (21). In the present study, D-GalN and LPS, which are well known for ALF induction (15,21-23), were used to induce an experimental acute liver injury model in mice. Consistent with previous results (15), the present results suggested that D-GalN/LPS significantly increased the levels of serum AST and ALT, enhanced the expression levels of the pro-inflammatory factors TNF-α and IL-6, and promoted hepatocyte apoptosis. Moreover, these results indicated that the D-GalN/LPS-induced ALF model was successfully established. In addition, miR-214 was identified to be significantly decreased in the liver tissue of D-GalN/LPS-induced mice. However, in the present study, groups of mice treated with only D-GalN or only LPS were not conducted, which may be a limitation, and thus further examination in future studies is required. Moreover, Bax was identified to be a direct target of miR-214 in BNLCL2 cells. However, the association between miR-214 and Bax in other hepatocyte cell lines was not investigated in the present study. Therefore, this is a limitation of the present study, and must be elucidated in the future.

ALF is characterized by extensive hepatocyte apoptosis and necrosis (24). Furthermore, 2 major mechanisms of cell death, namely the death receptor pathway and the mitochondrial pathway, are involved in the progression of ALF, in which TNF serves a crucial role (25). The caspase family is a key contributor to cell apoptosis, and it has been reported that caspase-3 is significantly activated in the ALF model (26). In addition, apoptotic-associated proteins tightly control cell apoptosis (27). For example, members of the Bcl-2 family interact with members of the Bax subfamily to induce apoptosis signals and cause apoptosis (28). In a GalN/LPS-treated mouse model, Bcl-2 expression was significantly decreased, while Bax expression was increased (29). Consistent with
previous studies (15,21-23), the present results demonstrated
that D-GalN/LPS treatment significantly increased hepatocyte
apoptosis and increased the expression Bax in vivo and in vitro.

Ameliorated hepatocyte apoptosis has been shown to be a
key step in the mitigation of D-GalN/LPS-induced ALF (30).
Previous studies have also reported that miRNAs can regulate
the expression of pro-apoptotic and anti-apoptotic genes (31).
Moreover, it has been reported that miR-15b and miR-16
negatively adjust TNF-α-mediated liver cell apoptosis via
Bcl2 in severe liver failure (32). In addition, miR-24 regulates
the key apoptotic gene Bcl2-like protein 11 during ALF (15).
The present results indicated that miR-214 expression was
downregulated in the liver tissues from mice stimulated with
D-GalN/LPS. It has also been reported that miR-214 serves
a suppressive role on extrinsic cell death pathways, such as
tissue necrosis and autophagy (33). A previous study indicated
that miR-214 improves acute kidney injury in vivo by inhibiting
apoptosis (34). miR-214 has also been demonstrated to protect
cells from hypoxia/reoxygenation-induced damage and attenuates ischemia/reperfusion (I/R)-induced myocardial
injury via suppression of PTEN and Bcl-2 homology domain 3
(BH3)-only Pro-Protein expression levels, leading to decreases
in I/R-induced myocardial apoptosis (35). The results of
the present study demonstrated that miR-214 ameliorated
D-GalN/TNF-α-induced cell apoptosis via targeting Bax.
Moreover, the specific association between these miRNAs,
including miR-214, miR-24, miR-15b and miR-16, in liver
injury requires further research.

In conclusion, the present results suggested that miR-214
was downregulated in a D-GalN/LPS-induced murine ALF model and in D-GalN/TNF-α-stimulated hepatocytes.
Moreover, it was identified that miR-214 ameliorated
D-GalN/TNF-α-induced inflammation and apoptosis in
hepatocytes via targeting Bax. Therefore, it was hypothesized
that miR-214 may serve as a novel therapeutic strategy for
ALF treatment. However, this is only a preliminary study on
the role of miR-214 in ALF, and the present study had
limitations; therefore, additional in-depth research is required to
establish the role of miR-214 in ALF. For example, the effects
of miR-214, on factors other than cell apoptosis, such as
hepatocyte function and stress markers in hepatocytes, should
be subsequently investigated. In addition, the present study
focused on the effect of miR-214 on normal murine embryonic
liver cells. However, due to the interspecies variation in hepatic

Figure 7. miR-214 decreases TNF-α and IL-6 levels in D-GalN/TNF-α-stimulated hepatocytes. BNLCL2 cells were transfected with mimic control, miR-214 mimic or miR-214 mimic + Bax plasmid for 24 h, then cells were treated with D-GalN (1 mg/ml) and TNF-α (100 ng/ml) for 36 h. (A) TNF-α and (B) IL-6 mRNA expression levels were measured by reverse transcription-quantitative polymerase chain reaction. Release of (C) TNF-α and (D) IL-6 in the culture medium of BNLCL2 cells was detected using ELISA. Data are presented as the mean ± standard deviation. *P<0.01 vs. control. **P<0.01 vs. model; ***P<0.01
vs. mimic. D-GalN, D-galactosamine; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; IL, interleukin; miR, microRNA.
responses, it is necessary to study the effect of miR-214 on human hepatocytes; this was also a limitation of the present study, which will be addressed in future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SW contributed to study design, data collection, statistical analysis, data interpretation and manuscript preparation. XH, WS, LC, YH, YW, EL, AQ and WZ contributed to data collection and statistical analysis. JG contributed to data collection, statistical analysis and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal care and animal experimental protocols were carried out strictly according to the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health and the Animal Ethics Committee of The First Affiliated Hospital of Suzhou University. The present study was approved by the Animal Ethics Committee of The First Affiliated Hospital of Suzhou University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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