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ORIGINAL ARTICLE

MiR-218-XOR-ROS Pathway Regulates the Progression of Nonalcoholic Steatohepatitis

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SUMMARY

Background: **To investigate the role of the miR-218-xanthine oxidoreductase (XOR) pathway in the pathogenesis of nonalcoholic steatohepatitis (NASH) and to explore the potential downstream mechanisms involving oxidative stress and energy metabolism.**
Methods: **The NASH animal model was established by feeding BALB/c mice with an MCD diet, while BRL-3A cells were cultured with a mixture of oleate and palmitate for 72 hours to mimic the steatosis and inflammation of NASH *in vitro*. The steatosis and inflammation levels were assessed by H-E/oil-red staining and serum/supernatant TG, ALT, and AST levels. The apoptosis degree was tested by the TUNEL/flow cytometry method both in animals and cultured cells. The XOR and miR-218 levels were detected by western blotting and qRT-PCR.**
Results: **Decreased miR-218 and increased XOR levels were identified in the NASH animal and cell models, while the regulation of miR-218 on XOR was also confirmed. NASH alleviation was achieved after miR-218 over-expression *in vivo* and *in vitro*, according to the declination of steatosis and inflammation-related markers. Although H_2O_2 and ATP levels were increased and decreased in NASH models, respectively, antagonizing miR-218 could significantly alleviate those changes.**
Conclusions: **The miR-218-XOR pathway may provide a novel mechanism and treatment option for NASH.**
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KEY WORDS

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is defined as diffuse hepatocyte steatosis with/without inflammation, precluding the overconsumption of alcohol and other clearly identified hepatic injury factors [1]. NAFLD is closely associated with diabetes and cardiovascular disease and is considered as the hepatic manifestation of metabolic syndrome. Currently, the prevalence of NAFLD has been increasing, reaching 15% in China [2] and 20% in some western countries [3]. Of all the well-

identified NAFLD stages, nonalcoholic steatohepatitis (NASH) is considered the "watershed" for its role as the major cause of cryptogenic cirrhosis and its high percentage of progression into liver fibrosis (41%) and end-stage liver diseases (5.4%) [4]. Therefore, it is of clinical importance to explore NASH in-depth.

Currently, although the "two hit" hypothesis has become the mainstream pathogenesis of NAFLD [5], the detailed mechanism of NASH remains unclear. Among the events comprising the "second hit", insulin resistance enhances hepatic free fatty acid intake and mitochondrial β oxidation [6], hence increasing the production of reactive oxygen species (ROS). ROS then react with unsaturated fatty acids on membrane phospholipid and produce lipid peroxidation to inhibit the electron transport in the mitochondrial respiratory chain. Such a reaction may form a vicious cycle and become an important step in the pathogenesis and progression of NASH. Currently, accumulated evidence has shown the important role of xanthine oxidoreductase (XOR) in ROS [7,8]. XOR belongs to the family of molybdenum dehydrogenase and is the rate-limiting enzyme of purine metabolism. Under normal physiological status, the liver has the highest XOR level of all human organs, indicating its important role in liver metabolism [9].

MicroRNA (miRNA) belongs to a family of non-coding RNAs with a length of 19 - 25 nucleotides. Mature miRNA is processed from a double-stranded hairpin precursor by the RNase III family member Dicer in the cytoplasm [10]. It recognizes the 3' untranslated region of target mRNAs with imperfect complementarity, leading to translational repression in mammals and mRNA cleavage in plants [11]. The expression pattern of miRNAs as disease biomarkers have been widely reported, including our reports in different stages of NAFLD and transition from simple steatosis into NASH [12,13]. Furthermore, the important role of the miRNA-target gene pair in the pathogenesis and progression of certain diseases has been gradually revealed, including NAFLD and inflammatory bowel disease [14].

Considering the importance of ROS in NASH progression, the possible involvement of XOR is also anticipated. However, the effect of XOR in NASH has been rarely studied, and our group is the only one to report significantly increased serum XOR levels in NAFLD patients [15]. According to the repression capacity of miRNA, the potential upstream miRNA levels of XOR should be decreased. Furthermore, based on previously reported significantly decreased miRNAs, we found miR-218 as the predicted miRNA of XOR according to the bioinformatics-related prediction algorithm. Therefore, in this study, for the first time, we verified the regulatory role of miR-218 in XOR, as well as reported the effect of the miR-218-XOR-ROS pathway in NASH and potential underlying mechanisms.

MATERIALS AND METHODS

Ethics statement

This study was performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol using animals was approved by the institutional review board of the First Affiliated Hospital of Zhejiang University.

Dual luciferase reporter system construction and cell transfection

First, the gene fragment containing the 3' UTR region of the XOR wild-type (CAAGCACAT)/mutant (TCCA TCTCG) (Figure 1) and the specific dual-luciferase miRNA target expression vector pmirGLO (Promega, USA) were cut with the Sac I and Sal I restriction enzymes. There after, the samples were incubated with T4 DNA ligase (Fermentas, Lithuania) at 22°C for 30 minutes. Second, the pmirGLO-XOR-3'UTR vectors were used to transfect competent cells with $CaCl_2$. The transfected cells were then cultured at 37°C for 16 hours, followed by vector extraction (TIANGEN, China) and verification by gene sequencing. Third, the miR-218 mimics/control miRNA mimics (miR-NC) were synthesized (Figure 2). HEK-293T cells were transfected with the pmirGLO-XOR-3'UTR vector and above-mentioned miRNAs using Lipofectamine 2000 (Invitrogen, USA). After culturing at 37°C/5% CO_2 for 24 hours, the co-transfected cells were harvested, and the luciferase activity was tested using a dual-luciferase reporter gene detection kit (Promega, USA). In this step, subjects were divided into five groups as follows: control group, XOR-wild type (WT) + miRNA mimic group, XOR-WT + miRNA-218 mimic group, XOR-mutant (Mut) + miRNA mimic group, and XOR-Mut + miRNA-218 mimic group.

Construction of the NASH animal and cell models with different treatments

In total, 24 male BALB/c mice aged 6 weeks were purchased from Cavens Lab Animal (Suzhou, China) and randomly divided into four groups: Control + saline (n = 6), NASH + saline (n = 6), NASH + Agomir-NC (n = 6), and NASH + Agomir-218 (n = 6). All mice received food and water ad libitum and were maintained on a 12/12-hour light/dark cycle. The Control + saline group was given a basic diet, while the NASH group was given an MCD diet for 4 weeks as previously reported [20]. Saline, miRNA NC, and miR-218 mimic were respectively given to the NASH + saline group, NASH + Agomir-NC group, and NASH + Agomir-218 group (the same sequence as shown in Figure 2 but with a different chemical modification). The mice were sacrificed by neck dislocation at the appointed time with blood and liver tissue collection. The liver sections were then stained with haematoxylin-eosin (H-E) and were observed for hepatic steatosis and inflammation using an Olympus microscope, with the histological activation

index (HAI) calculation as the semi-quantitative marker of the severity of hepatic injury [21]. Furthermore, the lipid deposition-related marker triglyceride (TG) and inflammation-related marker alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were detected using a Hitachi 7600 clinical analyser (Department of Laboratory, the First Affiliated Hospital of Zhejiang province). ROS production was measured by the DCFH-DA fluorescent probe method, according to the manufacturer's instructions (Beyotime Biotechnology Ltd., China).

BRL-3A cells were purchased from Fu-xiang Biotechnology Ltd. (Shanghai, China) and were cultured grown under routine culture conditions of 5% CO_2/95% air at 37°C. Before incubation, cell viability was tested by Trypan blue exclusion, and viability greater than 90% was considered eligible. Eligible BRL-3A cells with at least 80% confluency were exposed to HFFA, a mixture of oleate (OA) and palmitate (PA), at the final ratio of 2:1 and final concentration of 1 mM for 72 hours. They were then harvested for the detection of steatosis and inflammation. The control and NASH groups were further treated with miR-NC, miR-218 mimic, siRNA-Scr, and siRNA-XOR to evaluate the effect of miR-218 over-expression and XOR down-regulation. For lipid droplet observation, the harvested BRL-3A cells were rinsed with PBS and fixed with 10% neutral formalin for 30 minutes. They were then dyed with Oil red (1 mg/mL in PBS) at 37°C for 20 minutes and re-rinsed with PBS before microscope observation. Other steatosis-, inflammation- and oxidative-related markers detected in the BRL-3A cell supernatant were the same as those in the NASH animal model.

***In vitro* and *in vivo* apoptosis test**

Apoptosis in BRL-3A cells with different treatments (control + miR-NC, control + miR-218 mimic, control + siRNA-Scr, control + siRNA-XOR, NASH + miR-NC, NASH + miR-218 mimic, NASH + siRNA-Scr, NASH + siRNA-XOR) was analysed using Annexin V-EGFP/PI double-staining method by flow cytometry according to the manufacturer's instructions (Shanghai R&S Biotechnology Co., Ltd., China). Cells from the NASH and control groups were digested by trypsin, centrifuged at 1,000 rpm for 5 minutes, washed in PBS twice and re-suspended in 400 μL of binding buffer. Thereafter, 5 μL of Annexin V-FITC and 10 μL of PI were consecutively added and incubated in the dark for 30 minutes at 20°C. Apoptosis was then assessed by dual-color flow cytometry on a FACScan cytofluorometer (BD Bioscience) using Cell Quest software (BD Bioscience). Additionally, apoptosis in the control and NASH animal models (control + saline, NASH + saline, NASH + Agomir-NC, and NASH + Agomir-218) were carried out by the routine TUNEL method (Roche, USA), where apoptosis index = (apoptotic cell/total cell) * 100%.

***In vivo* and *In vitro* RNA interference**

Briefly, miRNA-218 mimic and miRNA-NC were routinely synthesized as mentioned above. BRL-3A cells cultured with HFFA and control liquid were transfected with different regents using Lip-2000. Previously synthesized AgomiR-218 and AgomiR-NC were used as in vivo siRNAs after cholesterol modification and HPLC purification. In detail, the caudal veins of 32 BALB/c mice were injected twice every week with different reagents: control + Saline, NASH + Saline, NASH + AgomiR-NC, and NASH + AgomiR-218. After 4 weeks of feeding, the mice were sacrificed at 24 hours after the last caudal vein injection. Their liver tissue and blood were collected for further analysis.

Statistical analyses

Statistical analyses were performed using SPSS, version 16 (SPSS, Chicago, IL, USA). The data are presented as means ± standard deviation when the data were found to be normally distributed or as medians if the distribution was skewed. Differences between groups were analysed using Student's *t*-test or the Mann-Whitney *U* test.

RESULTS

Significantly changed XOR levels in successfully established NASH models

The NASH mouse model was successfully established by MCD diet feeding as confirmed by hepatic fat deposition, hepatocyte ballooning, and mild to moderate chronic portal and intra-acinar inflammation (Figure 1A) as well as the increased apoptosis degree in NASH group (Figure 1B). The NASH cell model was successfully constructed by culturing BRL-3A cells with HFFA for 72 hours as confirmed by hepatocyte lipid deposition (Figure 1C). Furthermore, elevated ALT, AST, and TG levels were identified both in NASH animal and cell models. Moreover, the XOR level was significantly increased in both NASH cells and the animal models as shown by Western blotting.

Decreased miR-218 level in NASH and its regulatory role on XOR

Since miR-218 was predicted as the up regulator of XOR by bioinformatics (Figure 2A), we started to investigate its level in NASH. The significantly decreased miR-218 level was identified in the NASH animal model using the qRT-PCR method (Figure 2B) while over-expressing miR-218 could significantly decrease XOR levels in both cell (Figure 1D) and animal (Figure 1E) models. Further, the dual luciferase reporter system showed significant down regulation of the Firefly/Renilla level in the XOR-WT + miRNA-218 mimic group compared with that in the XOR-WT + miRNA mimic NC group, confirming the regulation of miRNA-218 on XOR (Figure 2C).

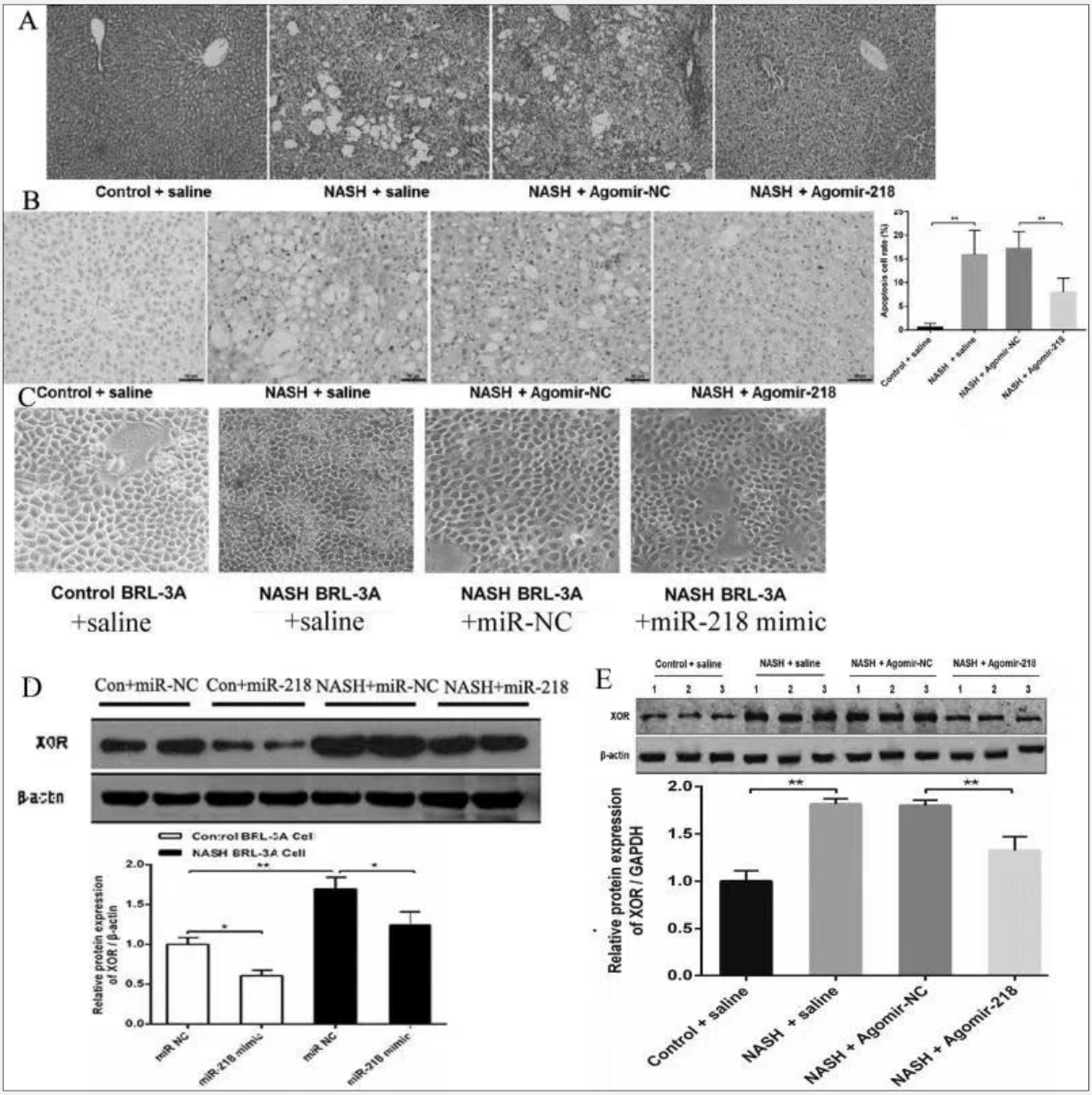

Figure 1. Change of XOR level in successfully established NASH model.

(A) Liver H-E staining in different groups; (B) Semi-quantification of the apoptosis degree in the different groups as indicated by the accumulation of cells and particles; (C) Oil red staining showed the lipid deposition degree changes after antagonizing miR-218 level; western blotting showed increased XOR levels in NASH cell (D) and animal (E) groups, while miR-218 over-expression significantly decreased the XOR level.

Effects of antagonizing miR-218 in the successfully established NASH animal and cell models

Over-expressing miR-218 in NASH mouse model significantly alleviated the liver yellowish change and enlargement according to the morphologic examination and decreased hepatocyte lipid deposition and intra-acinar inflammation according to H-E staining analysis, compared with that in both the NASH + saline group and NASH + Agomir-NC group (Figure 1A). In addition, the *in vivo* study showed that the apoptosis degree (Figure 1B) and ALT, AST, and TG levels were significantly decreased after successful miR-218 over-expres-

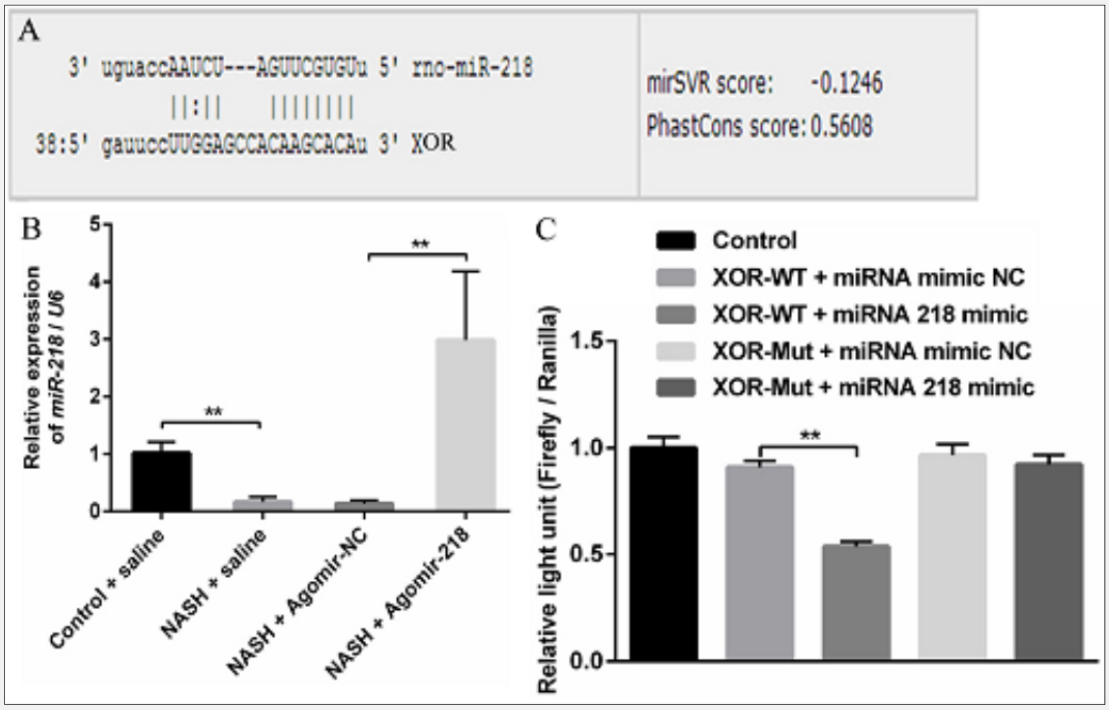

Figure 2. Significant change of miR-218 in NASH animal model and its regulatory role on XOR.

(A) Bioinformatics predicts the binding site between miR-218 and XOR; (B) Significantly changed miR-218 level in NASH and after its over-expression; (C) The luciferase reporter system shows the regulation of miR-218 on XOR.

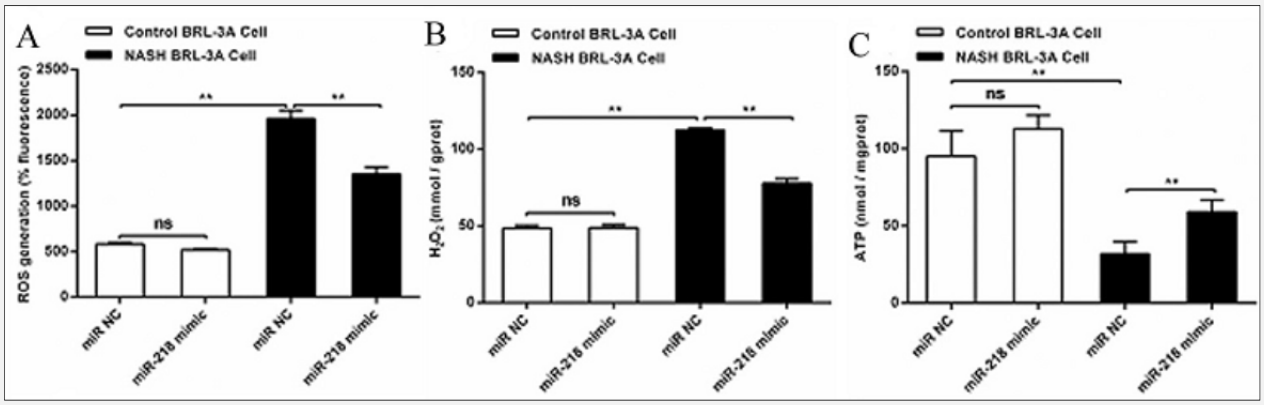

Figure 3. Significantly altered change of ROS production rate (A), H_2O_2 (B), and ATP (C) levels in NASH cell model, with and without miR-218 over-expression.

sion compared with those in the groups treated with saline and Agomir-NC. Similar results were also identified in *in vitro* studies, where apoptosis and the lipid deposition degree were significantly decreased in NASH BRL-3A cells treated with miR-218 mimic compared with those treated with miR-NC (Figure 1C). The ALT, AST, and TG levels were also significantly decreased in the miR-218 mimic-treated NASH BRL-3A cells compared with those treated with miR-NC. All these results showed the capacity of miRNA-218 in alleviating NASH progression at the *in vivo* and *in vitro* levels.

Potential downstream mechanism of the miR-218-XOR pathway in NASH

Since miR-218 interference has a significant effect on NASH and the regulatory role of miR-218 on XOR was confirmed, we further investigated the potential downstream molecules. We found that ROS generation rate, H_2O_2 and ATP levels were significantly changed in the NASH cell model. Furthermore, miR-218 mimic could significantly antagonize such changes (Figure 3).

DISCUSSION

Currently, the pathogenesis and progression of NASH, in which mitochondrial dysfunction and ensuing intensified oxidative stress are advocated, is vague. In this study, we found a significant increment in XOR in both the NASH animal and cell models, supporting the potential pathological role of XOR in NASH. This hypothesis is theoretically plausible for the following reasons. First, XOR can decompose ATP and other purine nucleotides into uric acid, which plays a pivotal role in NAFLD [16,17]. In detail, abundant active oxygen radicals are produced during the process of uric acid synthesis under XOR catalysis, which further participates in tissue oxidative damage [18]. Second, the animal study showed that XOR-induced ROS accumulation is the direct reason for tissue damage, while XOR activity suppression by allopurinol could significantly alleviate ischaemia/reperfusion damage [19]. Third, a previous study demonstrated the significantly increased XOR level in viral liver cirrhosis and its positive association with inflammation [20]. Finally, further research showed that the XOR level and activity in steatotic hepatocytes are significantly increased, while XOR suppression by allopurinol could alleviate oxidative stress-induced liver injury [20].

Based on the effect of XOR in NASH, we searched for its upstream regulator. Through bioinformatics prediction and the ensuing dual luciferase reporter system verification, we, for the first time, confirmed the regulatory role of miR-218 on XOR. Further overexpression of miR-218 could significantly decrease the XOR level in control and NASH cell models. A previous study has already reported the importance of miR-218 in tumorigenesis and metastasis, acting as a tumour suppressor by targeting many oncogenes related to proliferation, apoptosis, and invasion [14]. Furthermore, a significantly decreased serum miR-218 level in patients with hepatocellular carcinoma was also reported [21], but its effect in NASH remains blank. The miR-218-target pathways have been identified in various cancers, including hexokinase 2 in glioma [22], diphthamide biosynthesis 1 in colorectal cancer, and ROBO1/RET in liver cancer [23-25]. Nevertheless, the miR-218-XOR pathway in NASH has not been reported hitherto, and our results indicated the potential therapeutic effect of this pathway in NASH.

The downstream pathway of miR-218-target mRNA has been reported in various diseases and include the PRKCE/MDR1 axis in gallbladder cancer, CDK6/CyclinD1/E2F1 axis in gastric cancer, and WNT/β-Catenin axis in ovarian cancer [26,27]. Nevertheless, oxidative stress and energy metabolism as its downstream effectors have rarely been reported. In this study, we found that antagonizing miR-218 could significantly change the ATP and H_2O_2 levels, supporting their role as downstream effectors of the miR-218-XOR axis. However, as ATP and H_2O_2 participate in many physiological and pathological processes, whether their changes are only attributed to the miR-218-XOR pathway or are the result of NASH alleviation should be considered cautiously and need further careful exploration.

Several limitations of this study should be acknowledged. First, we only detected the level of XOR in NASH animal and cell models. The effect of antagonizing XOR in NASH needs further investigation. Second, although the regulatory role of miR-218 on XOR was confirmed by the dual luciferase reporter system, it is better to consolidate such an effect using RNA-FISH that is also used to show the location of the combination. Third, since one miRNA may regulate hundreds of downstream mRNAs, there might be many other downstream molecules of miR-218 that participate in NASH pathogenesis that need further study. Finally, our results were derived only from the animal and cellular levels; whether these results could be generalized to humans needs further research.

CONCLUSION

In summary, our study reported the significantly dysregulated miR-218-XOR pathway in NASH. Further study also revealed the potential therapeutic effect of targeting miR-218 in NASH and the involvement of ATP and H_2O_2 as a downstream mechanism. Our results provide a novel mechanism and potential method for the pathogenesis and treatment of NASH.

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Authors' Contribution:
Junling He and Juanwen Zhang carried out the microarray analysis and drafted the manuscript; Mosang Yu participated in the design of the study and performed the bioinformatics analysis and qRT-PCR; Yue Huang performed all laboratory tests and was involved in the statistical analysis. Yuying Dai and Ruoheng Zheng conceived of the study and supervised in its design and coordination.

Declaration of Interest:
There are no conflicts of interest or commercial financial support for this manuscript.

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