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miR-222 targets ACOX1, promotes triglyceride accumulation in hepatocytes

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent chronic liver diseases. However, the exact pathogenesis of NAFLD remains to be elucidated. Despite the association with tumors and cardiovascular diseases, the role of miR-222 in NAFLD remains unclear. The present study was to investigate the role of miR-222 in NAFLD.

Methods: Wild-type C57BL/6 mice were fed a high-fat diet for 12 weeks to induce NAFLD. Normal human liver cell line (L02) was cultured with free fatty acid (FFA)-containing medium to stimulate cell steatosis. The mRNA levels of miR-222 and acyl Coenzyme A xidase 1 (ACOX1) were detected by quantitative-PCR (Q-PCR). The prediction of ACOX1 as the target gene for miR-222 was conducted via TargetScan. The overexpression or inhibition of miR-222 was mediated by miR-222 mimics or antagomir, and intracellular triglyceride levels were measured using a triglyceride kit. Luciferase reporter assays verified ACOX1 as the target gene for miR-222.

Results: miR-222 was significantly elevated in both the *in vivo* and *in vitro* NAFLD models. Overexpression of miR-222 significantly increased triglyceride content in the L02 cells, while inhibition of miR-222 expression restricted the accumulation of triglyceride. Overexpression of miR-222 significantly inhibited ACOX1 expression. Transient transfection assays verified that ACOX1 3′-UTR luciferase reporter activity could be inhibited by miR-222 overexpression.

Conclusions: The present study suggested that miR-222 promotes the accumulation of triglycerides by inhibiting ACOX1.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by excess accumulation of triglycerides (TG) in the liver of patients without a prior history of excessive alcohol consumption. The prevalence of NAFLD is approximately 20% in China and even higher in industrialized countries [1]. NAFLD includes simple fatty liver (SFL), non-alcoholic steatohepatitis (NASH), and related cirrhosis. Poorly controlled SFL can further progress to NASH, liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC) [2,3]. NAFLD is projected to be the most important pathology for liver transplantation in the next decade [4]. However, the pathogenesis of fatty liver remains unclear. The current accepted doctrine is the 'two-hit' hypothesis. The theory states that the first hit is an excessive accumulation of lipids in the liver, while the second attack is hepatocellular damage, inflammation and liver fibrosis due to mitochondrial dysfunction [5]. However, the pathophysiological process of NAFLD is in fact a complicated and multistep process, characterized by multiple stimuli acting together on a genetically predisposed subject [6,7]. Since the pathogenesis of NAFLD and its corresponding therapeutic targets remain unclear, knowledge regarding the clinical treatment of NAFLD is sparse. Therefore, the unveiling of NAFLD pathogenesis is of utmost importance.

MicroRNAs (miRs) are a class of noncoding, endogenous, evolutionarily conserved, short-chain RNA, typically 21–25 bases in length. miRs exert posttranscriptional regulation by inhibiting and degrading the mRNA of the target gene [8], and play an important role in the pathological and physiological processes of development, stem cell differentiation, apoptosis, tumors, immune diseases, cardiovascular diseases, neurological diseases, and renal diseases. On the other hand, many miRs are involved in metabolic diseases, such as diabetes, NAFLD, obesity, and insulin resistance [9]. At present, frequently studied miRs in NAFLD include miR-122, miR-33, and miR-21. These miRs regulate different processes of lipid metabolism, including fatty acid synthesis, transport and oxidation. miRs are essential in the development of

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NAFLD. miR-222 is also an important miR that has been studied in tumors, cardiovascular diseases and other fields [10,11]. Recently, the role of miR-222 in liver diseases has gained increasing attention. The primary focus remains on liver fibrosis, while its role in fatty liver is rarely described. Using Stelic Animal Model (STAM) to simulate NASH-induced HCC, de Conti et al. found that miR-222 was significantly elevated during hepatic steatosis and NASH [12]. In addition, the expression of miR-222 was also significantly increased in the liver of mice with insulin resistance induced by a high fat and sucrose diet, while the important molecule insulin receptor substrate-1 (IRS-1) in the insulin pathway was directly regulated by miR-222 [13]. It is worth noting that IRS-1 plays a role in diabetes and is a very important molecule in the pathogenesis of NAFLD [14]. These findings suggest that miR-222 may possess a crucial role in the pathogenesis of NAFLD.

The GEO database (GSE13840) showed that miR-222 expression was significantly increased in the ob/ob mice group. In addition, miR-222 expression was also elevated in liver miRs in another microarray of C57BL/6 mice fed with a high-fat diet (HFD) compared to those on a normal diet (NC) (GSE65978). This finding further suggested that miR-222 may be involved in the progression of NAFLD, but its specific role in NAFLD has not been clearly defined. The present study aimed to evaluate the relationship between miR-222 and triglyceride accumulation in both *in vivo* and *in vitro* model, and to investigate the underlying mechanisms.

Methods

Animals

Six-week-old male mice (18–20 g) were used. Six mice were fed HFD (carbohydrates, 20.3%; protein, 18.1%; fat, 61.6%; D12492, Research Diets, New Brunswick, NJ, USA) for 12 weeks, and were considered as the HFD group. Six mice served as the negative control (NC) group were fed a normal chow (carbohydrates, 71.5%; protein, 18.3%; fat, 10.2%; D12450B, Research Diets) for 12 weeks.

Cell culture

L02 cells were purchased from the Chinese Academy of Sciences. L02 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco/Thermo, Waltham, MA, USA), containing penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS), in a 5% CO_2/water-saturated incubator at 37 °C. Steatosis was simulated by culturing in medium containing 1 mmol/L free fatty acids (FFA) (oleic acid:palmitic acid 2:1) for 24 h. Cells were cultured in medium containing FFA after transfection with either miR-222 mimics (Forward: AGCUACAUCUGGCUACUGGGU; Reverse: CCAGUAGCCAGAUGUAGCUUU) or miR-222 antagomir (ACCCAGUAGCCAGAUGUAGCU) miR NC (Forward: UUCUCCGAACGUGUCACGUTT; Reverse: ACGUGACACGUUCGGAGAATT) for 24 h. The mock group is a control group that only adds transfection reagent but does not mix any nucleic acid.

Quantitative real-time polymerase chain reaction (Q-PCR)

TRIzol (Invitrogen, Carlsbad, CA,USA) was used to extract total RNA from the cells, and the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis. The TaqMan miRNA assay kit (Life Technologies, Waltham, MA, USA) was used for the miRNA assay. Universal SYBR Green (QIAGEN, Duesseldorf, NRW, Germany) was used for the Q-PCR. The mRNA expression level was evaluated relative to the internal control (β-actin) using the $2^{-\Delta\Delta Ct}$ method.

β-actin-F (M): 5′-TCCAGCCTTCCTTCTTGGGTATG-3′
β-actin-R (M): 5′-CATCCTGTCAGCAATGCCTGGGTAC-3′
U6-F (M): 5′-AGAGAAGATTAGCATGGCCCCTG-3′
U6-R (M): 5′-GTGCAGGGTCCGAGGT-3′
U6-F (H): 5′-CGCTTCGGCAGCACATATAC-3′
U6-R (H): 5′-CAGGGGCCATGCTAATCTT-3′
miR-222-F (M): 5′-CCCTCAGTGGCTCAGTAG-3′
miR-222-R (M): 5′-CCACCAGAGACCCAGTAG-3′
miR-222-F (H): 5′-CGCAGCTACATCTGGCTACTG-3′
miR-222-R (H): 5′-GTGCAGGGTCCGAGGT-3′
ACOX1-F (H): 5′-GGCGCATACATGAAGGAGACCT-3′
ACOX1-R (H): 5′-AGGTGAAAGCCTTCAGTCCAGC-3′
Acox1-F (M): 5′-TAACTTCCTCACTCGAAGCCA-3′
Acox1-R (M): 5′-AGTTCCATGACCCATCTCTGTC-3′

Western blotting

Proteins were extracted from cells using RIPA Lysis Buffer (Beyotime, Shanghai, China) with Protease inhibitor cocktail for general use (Beyotime, Shanghai, China), then separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, MA, USA). After blocking with 5% bovine serum albumin in tris buffered saline with tween 20 (TBST), the membranes were incubated overnight with ACOX1 primary antibodies (1:1000, Proteintech, Chicago, IL, USA) at dilutions specified by the manufacturers. The membranes were then incubated with corresponding horse radish peroxidase (HRP)-conjugated secondary antibody at 37 °C at a 1:5000 dilution for 1 h. After washing 3 times with TBST, the signals were probed using an enhanced chemiluminescence (ECL) kit (Multisciences, Hangzhou, China). Image analysis was performed using the ImageQuantTL LAS 4000 mini gel imaging system. Images were generated using ImageJ software to determine the gray level of the target protein band. The ratio of the target protein to the internal reference protein β-actin was taken as the relative expression of protein.

Cellular triglyceride detection

Intracellular and intrahepatic TG contents were detected using kits purchased from Applygen Technologies Inc. (Beijing, China) in accordance with the vendor's recommended protocols.

Transient and dual luciferase reporter genes

Transient transfection and luciferase reporter assays were performed according to standard procedure. In short, L02 cells were transfected with the stated plasmids using transfection reagent lipofectamine 2000 (Thermo Fisher Scientific). Finally, a dual-luciferase reporter assay system (Promega, Madison, WI, USA) was used to measure the luciferase activity, and pRL-TK vector was used as a control to normalize luciferase activity.

Statistical analysis

Data were presented as the mean ± standard error of mean (SEM) of at least 3 independent experiments. Significant differences were analyzed with the indicated statistical methods using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

Results

The expression of miR-222 increased in both in vivo and in vitro NAFLD models

The body weight of the mice in the HFD group was greater than that of the NC group (Fig. 1B). Oil red O staining revealed high accumulation of red lipid droplets on the hepatocytes of the HFD group, while hematoxylin and eosin (H&E) staining showed

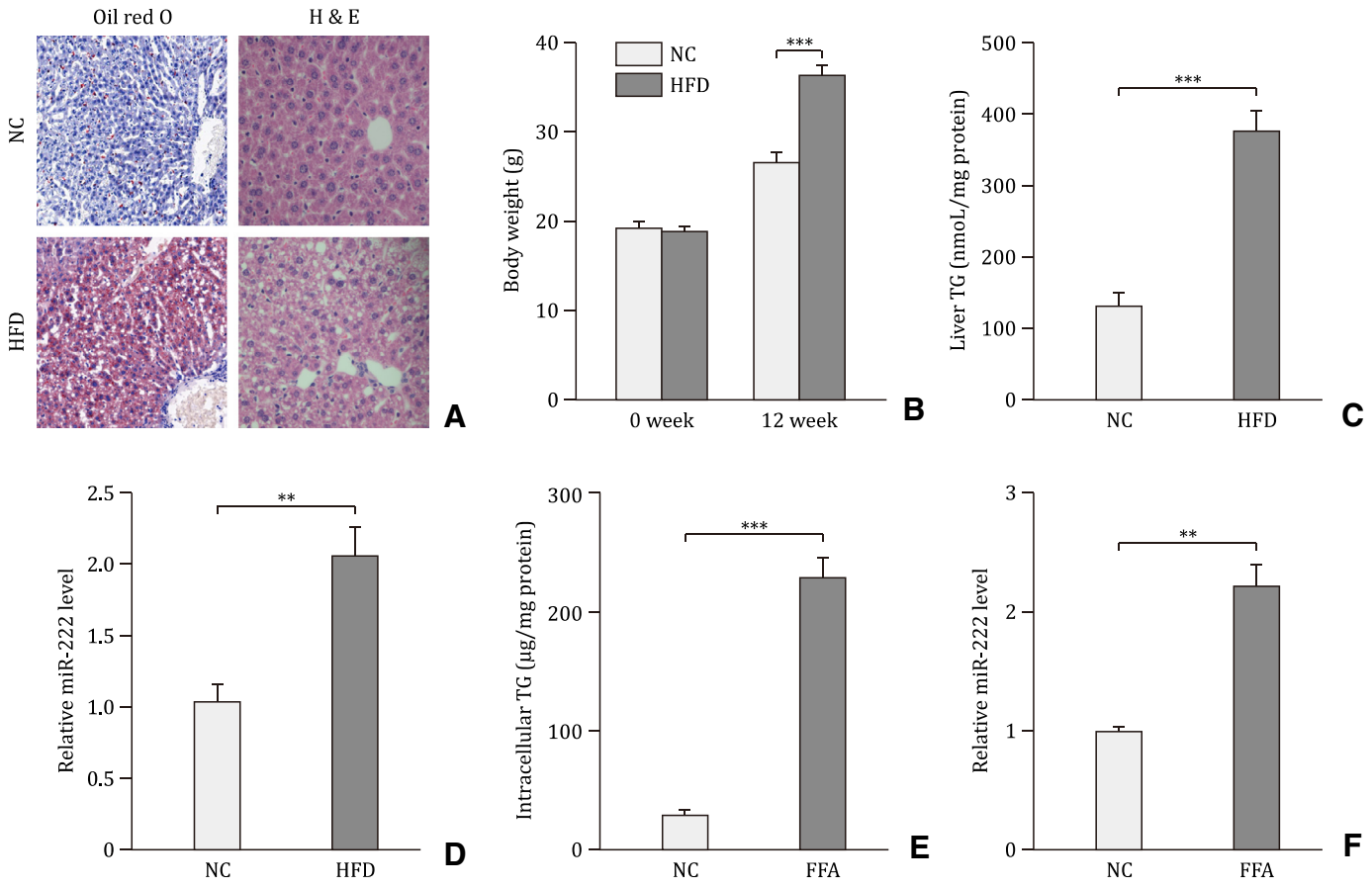

Fig. 1. Expression of miR-222 increased in both *in vivo* and *in vitro* NAFLD models. **A**: H & E staining and oil red O staining showed significant accumulation of TG and fatty liver formation in the HFD group (original magnification × 400); **B & C**: Body weight and intrahepatic TG content of the HFD group were higher than those in the NC group; **D**: miR-222 was significantly increased in HFD fed mice; **E** FFA significantly increased the TG content in L02 cells; **F**: miR-222 was significantly increased in FFA-treated L02 cells. Data are shown as mean ± SEM. **: $P < 0.01$; ***: $P < 0.001$. HFD: high-fat diet; NAFLD: non-alcoholic fatty liver disease; NC: negative control; TG: triglyceride; SEM: standard error of mean; FFA: free fatty acids.

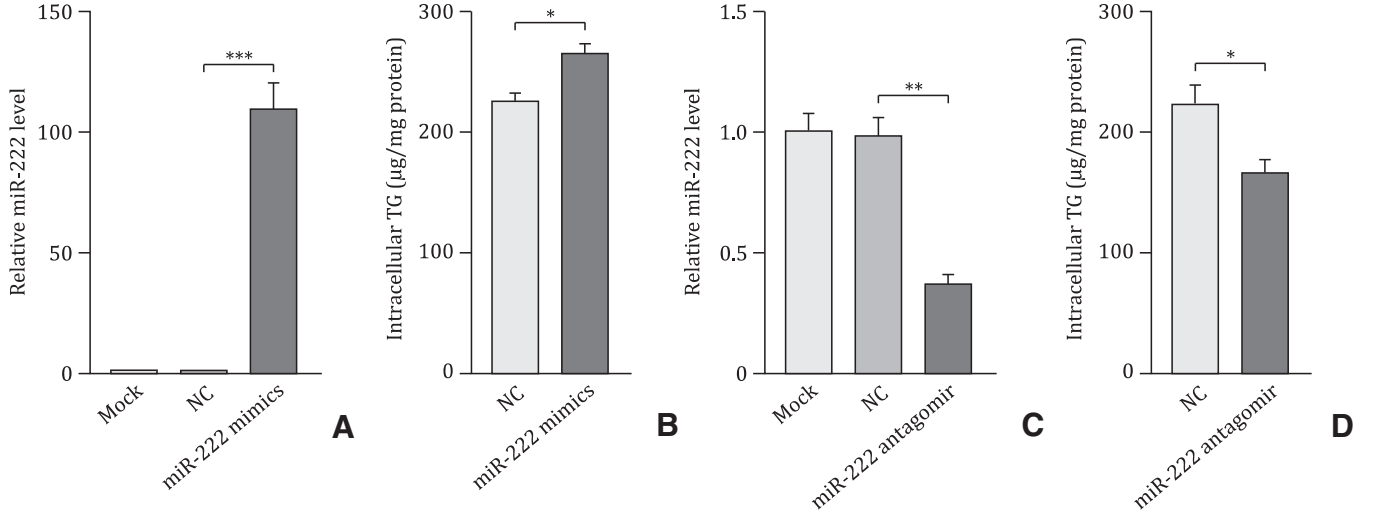

Fig. 2. miR-222 manipulation and cell TG content in L02 cells. **A**: Transient transfection of L02 cells with miR-222 mimics; **B**: miR-222 mimics increased TG content; **C**: miR-222 antagomir significantly decreased miR-222; **D**: miR-222 antagomir significantly decreased TG content. Data are shown as the mean ± SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. TG: triglyceride; NC: negative control; SEM: standard error of mean.

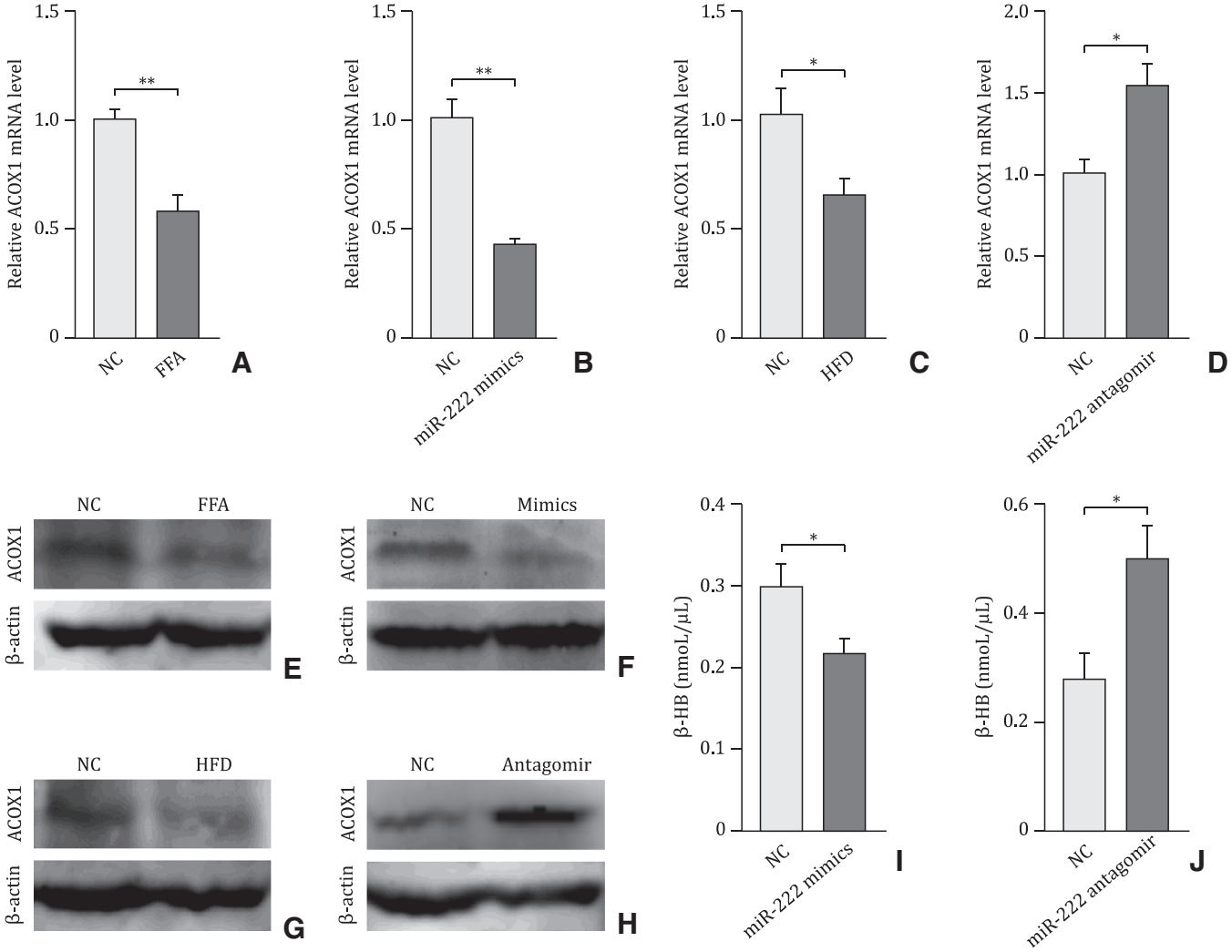

Fig. 3. The effects of miR-222 on ACOX1 and β-HB. ACOX1 mRNA in the NAFLD *in vitro* (**A**) and *in vivo* (**C**) models. ACOX1 protein in liver cell steatosis in the *in vitro* (**E**) and *in vivo* (**G**) models. The effect of miR-222 mimics (**B**) or miR-222 antagomir (**D**) on ACOX1 mRNA. The effect of miR-222 mimics (**F**) or miR-222 antagomir (**H**) on ACOX1 protein. The effect of miR-222 mimics (**I**) or miR-222 antagomir (**J**) on β-HB L02 cells cultured in FFA medium Data are shown as the mean ± SEM. *: $P < 0.05$; **: $P < 0.01$. ACOX1: acyl Coenzyme A xidase 1; β-HB: β-hydroxybutyrate; NAFLD: non-alcoholic fatty liver disease; NC: negative control; HFD: high-fat diet; SEM: standard error of mean; FFA: free fatty acids.

that the liver cells of the HFD group exhibited hepatocellular ballooning (Fig. 1A). The intrahepatic TG content of the mice in the HFD group was significantly higher than that of the NC group (Fig. 1C). miR-222 expression was significantly increased in mouse fatty liver tissue compared to the NC group (Fig. 1D). Cell experiment showed that FFA significantly increased intracellular TG content in L02 cells (Fig. 1E), which was parallel with the expression level of miR-222 ($P < 0.01$, Fig. 1F).

Changes of miR-222 in L02 cells affect TG accumulation

miR-222 mimics significantly promoted the accumulation of intracellular TG in steatosis L02 cells ($P<0.05$, Fig. 2A, B), while miR-222 antagomir significantly decreased TG content in steatosis L02 cells ($P < 0.05$, Fig. 2C, D).

miR-222 reduced ACOX1 expression and accumulation of β-hydroxybutyric acid (β-HB) in L02 cells

To investigate the specific mechanism of miR-222 on the metabolism of TG in hepatocytes, the target gene for miR-222 was predicted using the TargetScan website. ACOX1 was identified as a potential target gene for miR-222. The mRNA and protein levels of ACOX1 in both *in vivo* and *in vitro* NAFLD models were significantly lower than those of the NC group (Fig. 3A, C, E, and G). miR 222 antagomir significantly increased ACOX1 mRNA and protein *in vitro* NAFLD models (Fig. 3D, H). Furthermore, miR-222 mimics significantly downregulated the ACOX1 mRNA and protein levels (Fig. 3B, F). miR-222 overexpression significantly decreased the β-HB content in the supernatant of L02 cells (Fig. 3I). miR-222 antagomir significantly increased β-HB levels (Fig. 3J).

ACOX1 is the direct target of miR-222

We further predicted the binding sites of the 3′-UTRs of miR-222 and human (hACOX1) with TargetScan, which suggested two binding sites located at 306–312 (Fig. 4A) and 1818–1825 (Fig. 4B) of the ACOX1 3′ UTR, respectively. In addition, we separately mutated the 3′-UTR of ACOX1 binding to miR-222 (Fig. 4C, D). Overexpression of miR-222 inhibited the activity of the 3′-UTRs of ACOX1 in L02 cells (Fig. 4E, G), and mutation in one of these sites impaired the effect of miR-222 (Fig. 4F, H).

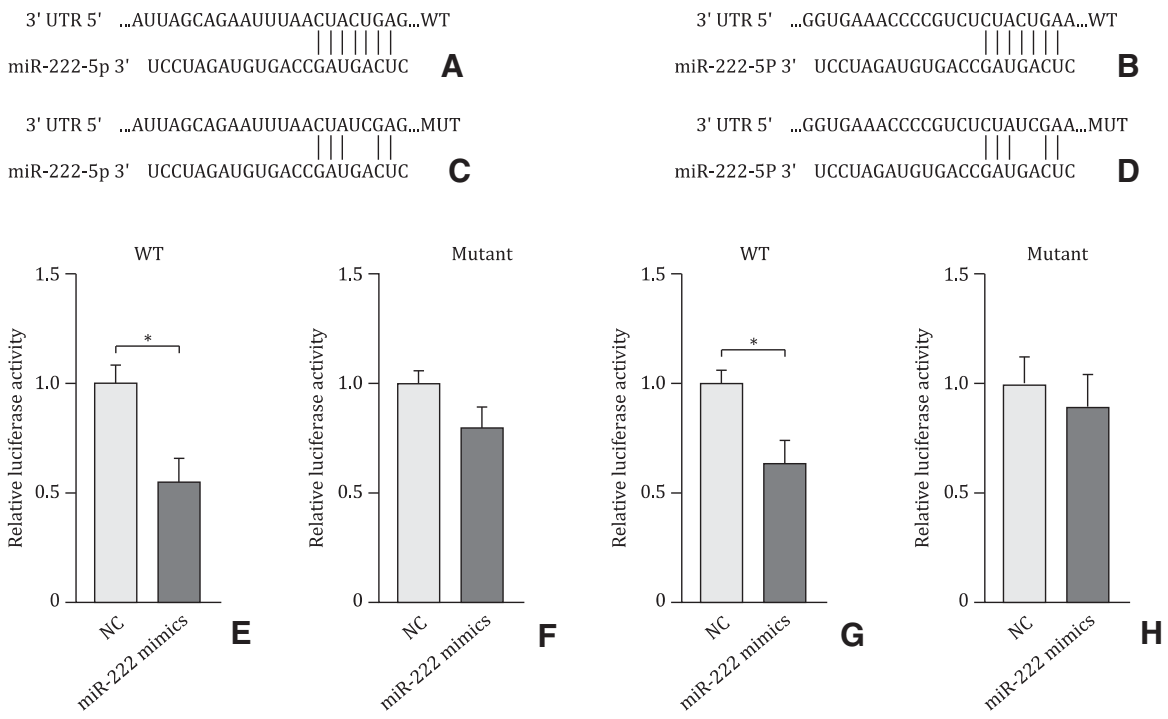

Fig. 4. miR-222 does not affect the levels of mutated ACOX1s. **A**, **B**: ACOX1 miRNA binding sites of miR-222; **C**, **D**: Mutated ACOX1 miRNAs do not bind miR-222 properly; **E–H**: miR-222 decreased the luciferase reporter activity in wildtype, not mutated ACOX1 in L02 cells. Data are shown as the mean ± SEM. *: $P < 0.05$. ACOX1: acyl Coenzyme A xidase 1; NC: negative control; WT: wild type.

Discussion

NAFLD is characterized by ectopic fat deposition in the liver, which is often associated with metabolic syndrome and abnormal glucose and lipid metabolism. In addition to inducing hepatic fibrosis and HCC, NASH is also associated with extrahepatic diseases (such as cardiovascular disease), thus its clinical importance has drawn increasing attention. The present study showed that miR-222 inhibited ACOX1, the key enzyme in fatty acid β-oxidation, thereby inhibiting β-oxidation of fatty acids and promoting accumulation of TG in liver cells. This finding elucidated a new mechanism for the pathogenesis of NAFLD. *In vitro* experiments showed that the use of miR-222 antagomir decreased intracellular TG content, which provided insight into a new potential NAFLD treatment target. miRs play an important role in gene regulation through posttranscriptional regulation [15]. The role of miR in NAFLD has received increasing attention [9]. However, there are many contradictions and ambiguities in miR and NAFLD-related studies, therefore, the study of miR and NAFLD warrants further investigation. miR-222 is an important miRNA and has a relevant role in the physiology and pathology of tumors, myocardial remodeling, angiogenesis, and viral myocarditis [10,11]. However, research on miR-222 in the liver has been primarily focused on liver cancer and hepatic fibrosis [16–18]. miR-222-3p and miR-221-3p were overexpressed in human HCC specimens and NASH-induced HCC models, in which miR-222-3p continues to rise at different stages of disease progression [19]. The role of miR-222 in hepatic fibrosis has also been fully recognized. However, the studies on miR-222 in fatty liver and other related metabolic diseases are sparse. miR-222 was significantly upregulated in the adipose tissue and peripheral circulation of obese patients and obese mice models [20,21]. Similar results have also appeared in diabetes studies. miR-222 was significantly elevated in the circulating blood of diabetic patients. The use of metformin and insulin can significantly reduce miR-222, while infusion of a fat emulsion/heparin mixture in patients significantly increased circulating miR-222 [22]. miR-222 was significantly increased in the livers of mice with insulin resistance induced by a high fat and high sucrose diet, and IRS-1, a crucial molecule in the insulin pathway, was the direct target of miR-222 [13]. There is no relevant study on miR-222 and hepatocyte steatosis. The current study aimed to fill the gap and partly explain the relationship between miR-222 and NAFLD.

ACOX1, the first rate-limiting enzyme of the fatty acid β-oxidation pathway, catalyzes the desaturation of acyl-CoAs to 2-trans-enoyl-CoAs. The β-oxidation of fatty acids can produce ketone bodies, including acetoacetate, β-HB and acetone. However, β-HB accounts for the highest proportion of ketone bodies [23]. ACOX1-deficient mice can develop severe microvesicular steatohepatitis, increase intrahepatic H_2O_2 levels and hepatocyte regeneration [24]. There are two subtypes of ACOX1: ACOX1a and ACOX1b. The use of viruses to drive ACOX1a or ACOX1b in ACOX1 knockout mice showed that ACOX1b was more effective than ACOX1a in reversing the phenotype of ACOX1 knockout mice [24]. Due to hepatocyte proliferation and persistent activation of peroxisome proliferator-activated receptor-mediated cytokines, aged ACOX1 knockout mice can develop spontaneous HCC [25,26]. miRNAs regulate ACOX1. ACOX1 was directly regulated by miR-15b-5p in colorectal cancer [27]. In human oral squamous cell carcinoma, miR-31-5p was also found to directly regulate ACOX1 [28]. Our study established that miR-222 can regulate ACOX1, which promoted the accumulation of TG in liver cells, and enriched the mechanisms of ACOX1 regulation.

Limitations of the study are mainly due to the lack of corresponding animal experiments. For instance, injections of miR-222 antagomir in NAFLD mice could be used to further confirm the efficacy of miR-222 inhibition for the treatment of NAFLD. In addition, the tissue samples of human NAFLD are difficult to obtain. Currently, we do not have relevant samples for NAFLD patients. However, we hope to confirm our results *in vivo* in the near future.

In conclusion, the present study confirmed that miR-222 can directly target ACOX1, a key enzyme for fatty acid oxidation, thereby inhibiting β-oxidation of fatty liver and promoting TG accumulation in hepatocytes. This study not only enriched the scientific knowledge concerning the pathogenesis of NAFLD but also provided new insight into the diagnosis and treatment of NAFLD.

Contributors

ZJ proposed the study. WJJ, ZYT and TYJ performed the research and wrote the first draft. All authors contributed to the design and interpretation of the study and to further drafts. ZJ is the guarantor.

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Ethical approval

All animal care and use procedures were conducted in accordance with regulations of the Department of Laboratory Animal Science at Fudan University (Grant No. 20160846A222).

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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