

Basic Study

miR-192-5p regulates lipid synthesis in non-alcoholic fatty liver disease through SCD-1

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Abstract**AIM**

To evaluate the levels of miR-192-5p in non-alcoholic fatty liver disease (NAFLD) models and demonstrate the role of miR-192-5p in lipid accumulation.

METHODS

Thirty Sprague Dawley rats were randomly divided into three groups, which were given a standard diet, a high-fat diet (HFD), and an HFD with injection of liraglutide. At the end of 16 weeks, hepatic miR-192-5p and stearoyl-CoA desaturase 1 (SCD-1) levels were measured. MiR-192-5p mimic and inhibitor and SCD-1 siRNA were transfected into Huh7 cells exposed to palmitic acid (PA). Lipid accumulation was evaluated by oil red O staining and triglyceride assays. Direct

interaction was validated by dual-luciferase reporter gene assays.

RESULTS

The HFD rats showed a 0.46-fold decrease and a 3.5-fold increase in hepatic miR-192-5p and SCD-1 protein levels compared with controls, respectively, which could be reversed after disease remission by liraglutide injection ($P < 0.01$). The Huh7 cells exposed to PA also showed down-regulation and up-regulation of miR-192-5p and SCD-1 protein levels, respectively ($P < 0.01$). Transfection with miR-192-5p mimic and inhibitor in Huh7 cells induced dramatic repression and promotion of SCD-1 protein levels, respectively ($P < 0.01$). Luciferase activity was suppressed and enhanced by miR-192-5p mimic and inhibitor, respectively, in wild-type SCD-1 ($P < 0.01$) but not in mutant SCD-1. MiR-192-5p overexpression reduced lipid accumulation significantly in PA-treated Huh7 cells, and SCD-1 siRNA transfection abrogated the lipid deposition aggravated by miR-192-5p inhibitor ($P < 0.01$).

CONCLUSION

This study demonstrates that miR-192-5p has a negative regulatory role in lipid synthesis, which is mediated through its direct regulation of SCD-1.

Key words: miR-192-5p; Stearoyl-CoA desaturase 1; High fat diet; Lipid synthesis; Non-alcoholic fatty liver disease

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Core tip: Hepatic miR-192-5p levels decreased in non-alcoholic steatohepatitis rat models fed a high-fat diet and the decrease could be reversed after disease remission by liraglutide therapy. miR-192-5p showed a direct interaction with stearoyl-CoA desaturase 1 (SCD-1). miR-192-5p overexpression significantly alleviated lipid accumulation in Huh7 cells exposed to PA, and SCD-1 siRNA abrogated the lipid deposition aggravated by miR-192-5p inhibitor. Our study provides evidence that miR-192-5p participates in lipid synthesis in non-alcoholic fatty liver disease (NAFLD) through SCD-1 and suggests that the overexpression of miR-192-5p may represent a promising treatment for NAFLD.

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INTRODUCTION

With the prevalence of obesity and metabolic syndrome, non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease worldwide, including China^[1]. Based on the "multiple hit" theory of NAFLD pathogenesis, lipid accumulation initiates simple hepatic steatosis and subsequently triggers multiple insults, ultimately inducing non-alcoholic steatohepatitis (NASH), cirrhosis, and even hepatocellular carcinoma in predisposed individuals^[2,3]. Briefly, high levels of lipid metabolites, such as free fatty acids, could cause mitochondrial dysfunction, endoplasmic reticulum stress, and consequent activation of inflammatory responses^[4,5]. In addition to the classical factors involved in the progression of NAFLD, epigenetic mechanisms are gradually identified as important regulators in the pathogenesis of this disease. The most thoroughly studied markers for epigenetic alterations in NAFLD are DNA methylation and the actions of microRNAs^[6-8]. MicroRNAs are non-coding RNAs composed of 18 to 25 nucleotides, and they play important roles in regulating a wide spectrum of biological processes, including fatty acid metabolism^[9,10]. Serum miR-192-5p levels have been reported to differentiate control livers, simple hepatic steatosis, and NASH in clinical studies^[11]. Similarly, our previous research in NAFLD patients also found that serum miR-192-5p levels showed good correlations with hepatic steatosis and inflammatory activity^[12]. Although miR-192-5p is abundant in the liver, early studies mainly focused on its regulatory role in cell growth, apoptosis, and tumor metastasis^[13,14], little is known about its role in lipid metabolism.

Stearoyl-CoA desaturase 1 (SCD-1) plays an important role in the biosynthesis of monounsaturated fatty acids and serves as a key regulatory enzyme in the last stage of hepatic *de novo* lipogenesis (DNL). Increased DNL has been confirmed in NAFLD patients compared with controls^[15]. Enhanced hepatic SCD-1 activity promotes the accumulation of hepatic lipids, especially triglyceride (TG), and consequently leads to the progression of NAFLD^[16]. Recent research has suggested that the expression of SCD-1 may be regulated through SREBP-1c-dependent and SREBP-1c-independent pathways^[17], but whether SCD-1 can be regulated by microRNAs in NAFLD has not been fully studied.

To address the above questions, we conducted this study in high-fat diet (HFD)-fed rats and palmitic acid (PA)-treated Huh7 cells. The hepatic and hepatocellular levels of miR-192-5p in NAFLD were evaluated both *in vivo* and *in vitro*. Overexpression and knockdown of miR-192-5p were performed in Huh7 cells to determine the regulatory effects of miR-192-5p in lipid

accumulation, and luciferase reporter assays were used to confirm the direct interaction between miR-192-5p and SCD-1. Collectively, we attempted to illustrate the role of miR-192-5p in hepatic lipid metabolism in NAFLD.

MATERIALS AND METHODS

Animals and treatment

The animal experiment was designed to minimize pain or discomfort to the animals. A total of 30 male Sprague-Dawley rats (6-wk-old) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and were housed under controlled conditions of temperature ($24\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$), humidity ($50\% \pm 5\%$), and a light/dark cycle (12 h) with free access to food and water. After acclimation for one week on a standard diet, they were randomized into three groups (10 rats/group). The control group received a standard diet; the HFD group was fed an HFD (88% standard diet, 10% lard, and 2% cholesterol); and the therapy group was fed an HFD and received intraperitoneal injections of liraglutide (Sigma, St. Louis, United States; 0.6 mg/kg in saline solution) for the last 8 wk. This experiment followed the National Research Council's Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of SHRM (SHRM-IACUC-001).

Sample collection and measurement

At the end of 16 wk, the rats were euthanized after an overnight fast. Parts of the rat livers were fixed in 4% paraformaldehyde overnight and embedded in paraffin for histological assessments with hematoxylin-eosin (H&E) staining. The remaining portions were snap frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ for oil red O staining and other analyses. The hepatic TG levels of the rats were measured with an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Cell culture

The Huh7 cell line was obtained from American Type Culture Collection (ATCC; Manassas, VA, United States) and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, United States) under an atmosphere of 5% CO_2 at $37\text{ }^{\circ}\text{C}$. PA powder (Sigma, St. Louis, United States) was dissolved in Milli-Q water supplemented with 1% fatty acid-free BSA (Sigma, St. Louis, United States) at $70\text{ }^{\circ}\text{C}$ and filtrated through a $0.22\text{-}\mu\text{m}$ filter to yield a 5 mmol/L stock solution of PA. The working PA solution was added to the cells at 0.3 mmol/L and 0.5 mmol/L.

Cell viability detection

We performed cell viability measurements using

the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Huh7 cells were plated onto a 96-well plate and 0, 0.3, and 0.5 mmol/L of PA in culture medium was added for 8, 16, and 24 h of incubation. The absorbance of the CCK-8 was read at 450 nm, and the values were normalized to those of the control group.

Oil red O staining and intracellular TG assay

For oil red O staining, the cell culture plate was washed twice with phosphate-buffered saline and fixed in 10% neutral formalin. Oil red O (Sigma, St. Louis, United States) was dissolved in isopropanol as a stock solution and was diluted 3:2 with ddH₂O to be added to the plate for 15 min, followed by washing in 60% isopropanol. Then, the plate was counterstained with hematoxylin after rinsing in distilled water. The intracellular TG levels in Huh7 cells were measured using a TG assay kit (Applygen Technologies Inc., Beijing, China) according to the manufacturer's instructions. Cellular TG levels were normalized to their protein contents.

MiRNA and small interfering RNA (siRNA) transfection

Huh7 cells were transfected with 50 nmol/L miR-192-5p mimic (Cat. No: miR10000222; Ribobio, Guangzhou, China), 200 nmol/L miR-192-5p inhibitor (Cat. No: miR20000222; Ribobio, Guangzhou, China), 50 nmol/L SCD-1 siRNA (Cat. No: stB0007776A; Ribobio, Guangzhou, China), and their respective negative control (NC; Ribobio, Guangzhou, China) according to the manufacturer's instructions with Lipofectamine 2000 (Invitrogen, Carlsbad, United States) and Opti-MEM medium (Gibco, CA, United States). The medium was replaced with DMEM with 10% FBS after transfection for 6 h. Experiments were performed 24 h after transfection.

Luciferase reporter assay

The sequence of the wild type (WT) or mutant (Mut) seed region of SCD-1 was cloned into a psiCHECK-2 luciferase vector (Promega, Madison, WI, United States) between XhoI and NotI sites. After being plated onto a 96-well plate, 293T cells were transfected with 0.16 μg of a SCD-1 3' untranslated region (UTR) vector (WT and Mut) and the empty vector as well as 50 nmol/L miR-192-5p mimic, 200 nmol/L inhibitor, and their respective NC. The culture medium was changed to complete DMEM after 6 h. Luciferase activity was measured using the Promega Dual-Luciferase system 48 h after transfection, and the relative luciferase activity was calculated as Renilla luciferase to Firefly luciferase.

Quantitative real-time polymerase chain reaction

Total RNA enriched with miRNA was isolated and extracted from frozen liver tissue and Huh7 cells

Table 1 Biochemical parameters of animal models

Parameter	Group		
	Control (n = 10)	HFD (n = 10)	Liraglutide (n = 10)
ALT, in U/L	37.4 ± 1.9	114.3 ± 26.7 ^a	34.3 ± 3.1 ^c
AST, in U/L	92.1 ± 5.6	160.1 ± 16.8 ^a	90.2 ± 5.5 ^c
HDL, in mmol/L	0.33 ± 0.01	0.25 ± 0.01 ^a	0.24 ± 0.01 ^d
LDL, in mmol/L	0.14 ± 0.01	0.42 ± 0.04 ^a	0.53 ± 0.07 ^a

Data are expressed as mean ± SEM. ^a*P* < 0.05 *vs* the control group; ^c*P* < 0.05 *vs* the HFD group. ALT: Alanine transaminase; AST: Aspartate transaminase; HDL: High density lipoprotein; LDL: Low density lipoprotein.

using TRIzol (Invitrogen, Carlsbad, CA, United States). RNA samples were analyzed on a NanoDrop 1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, United States) to assess its yield and purity. As previously described, complementary DNA for mRNA was synthesized using the PrimeScript RT Reagent Kit (Takara, Shiga, Japan) and SYBR Premix Ex Taq (Cat. No: RR420A; Takara, Shiga, Japan) was used for quantitative real-time polymerase chain reaction (qRT-PCR). In the reverse-transcription reactions of miRNA, poly A modification and first-strand cDNA synthesis were performed with 500 ng of total RNA each reaction using the Mir-X miRNA First-Strand Synthesis Kit (Cat. No: 638313; Takara, Shiga, Japan) according to the manufacturer's instructions. Subsequent qPCR was performed in a total reaction volume of 20 μL containing 2 μL of diluted (1:10) cDNA using SYBR Premix Ex Taq II (Cat. No: RR820A; Takara, Shiga, Japan) according to the manufacturer's instructions. Dissociation curve analysis was performed at the end of cycling program to control PCR specificity. The mRNA and miRNA abundance was normalized to that of 18 s and U6, and relative gene expression was analyzed based on the 2^{-ΔΔCt} method. Sequences of primers used were: SCD-1: 5'-GGATGCTCGTGCCAGTG-3', 5'-ACTCAGTGCCAGGTTAGAAG-3'; 18s: 5'-AAGTTTCAGCACATCCTGCGAGTA-3', 5'-TTGGTG A GGTCAATGTCTGCTTTC-3'; miR-192-5p: 5'-CTGACCTATGAATTGACAGCC-3'; and U6: 5'-AGA GAA GAT TAGCATGGCCCCTG-3'.

Western blot analysis

Protein samples of 30 μg were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBST for 2 h at room temperature and then incubated with mouse monoclonal antibody against SCD-1 (Abcam, Cambridge, United Kingdom) and mouse monoclonal tubulin antibody (Beyotime, Shanghai, China) overnight at 4 °C. Then, these membranes were washed and incubated at room temperature with an anti-mouse secondary antibody (Beyotime, Shanghai, China) for 1 h. Immune complexes were detected using a Western

chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, United States).

Statistical analysis

The data are expressed as the mean ± SEM. A statistical comparison was made using a two-tailed Student *t*-test between two groups and a one-way analysis of variance test followed by Student–Newman–Kuels analyses among multiple groups. Differences were considered significant at *P* < 0.05. All analyses were performed using GraphPad Prism 6.0 software (San Diego, CA, United States). The statistical methods used in this study were reviewed by Guang-Yu Chen from Clinical Epidemiology Center, Shanghai Jiao Tong University.

RESULTS

miR-192-5p and SCD-1 levels in the liver of rat models

At the end of the 16th week, all the rats in the HFD group developed NASH with significant hepatic macro-vesicular steatosis, ballooning degeneration, and lobular inflammation. The mean body weight of the HFD rats (661.7 ± 15.8 g) was higher than that of the controls (566.7 ± 8.1 g, *P* < 0.01), and they also had increased hepatic TG levels (335 ± 9 μmol/g) compared with the control group (101 ± 10 μmol/g, *P* < 0.01). The injection of liraglutide in HFD rats alleviated hepatic steatosis and reduced body weight (533.1 ± 7.4 g) and hepatic TG levels (241 ± 14 μmol/g) significantly (*P* < 0.01, Figure 1A-C). The analysis of serum biochemical parameters in animal models showed that the HFD rats had higher alanine transaminase (ALT), aspartate transaminase (AST), and low density lipoprotein (LDL) levels, and lower high density lipoprotein (HDL) levels compared with the control group (*P* < 0.05). The liraglutide group showed a significant decrease of ALT and AST compared with the HFD group (*P* < 0.05), but there was no statistical difference in serum LDL or HDL levels (Table 1). The qRT-PCR results showed that HFD rats had decreased hepatic miR-192-5p levels (0.46-fold) compared with the controls and that liraglutide therapy could abrogate the reduction of miR-192-5p in HFD rat livers (*P* < 0.01; Figure 1D). In addition to decreased hepatic miR-192-5p levels, the protein expression of hepatic SCD-1 was markedly elevated (3.5-fold) in rats fed an HFD, and liraglutide therapy could reduce hepatic SCD-1 levels in HFD rats (*P* < 0.01; Figure 1E).

Palmitate induces the down-regulation of miR-192-5p in vitro

According to the results of the CCK-8 test, 0.5 mmol/L PA induced cell death as early as 8 h, but 0.3 mmol/L PA showed no significant cytotoxic effects until 16 h (Figure 2A). To induce lipid accumulation without cytotoxicity *in vitro*, we chose to expose Huh7 cells to 0.3 mmol/L PA for 8 h. The TG levels in Huh7 cells

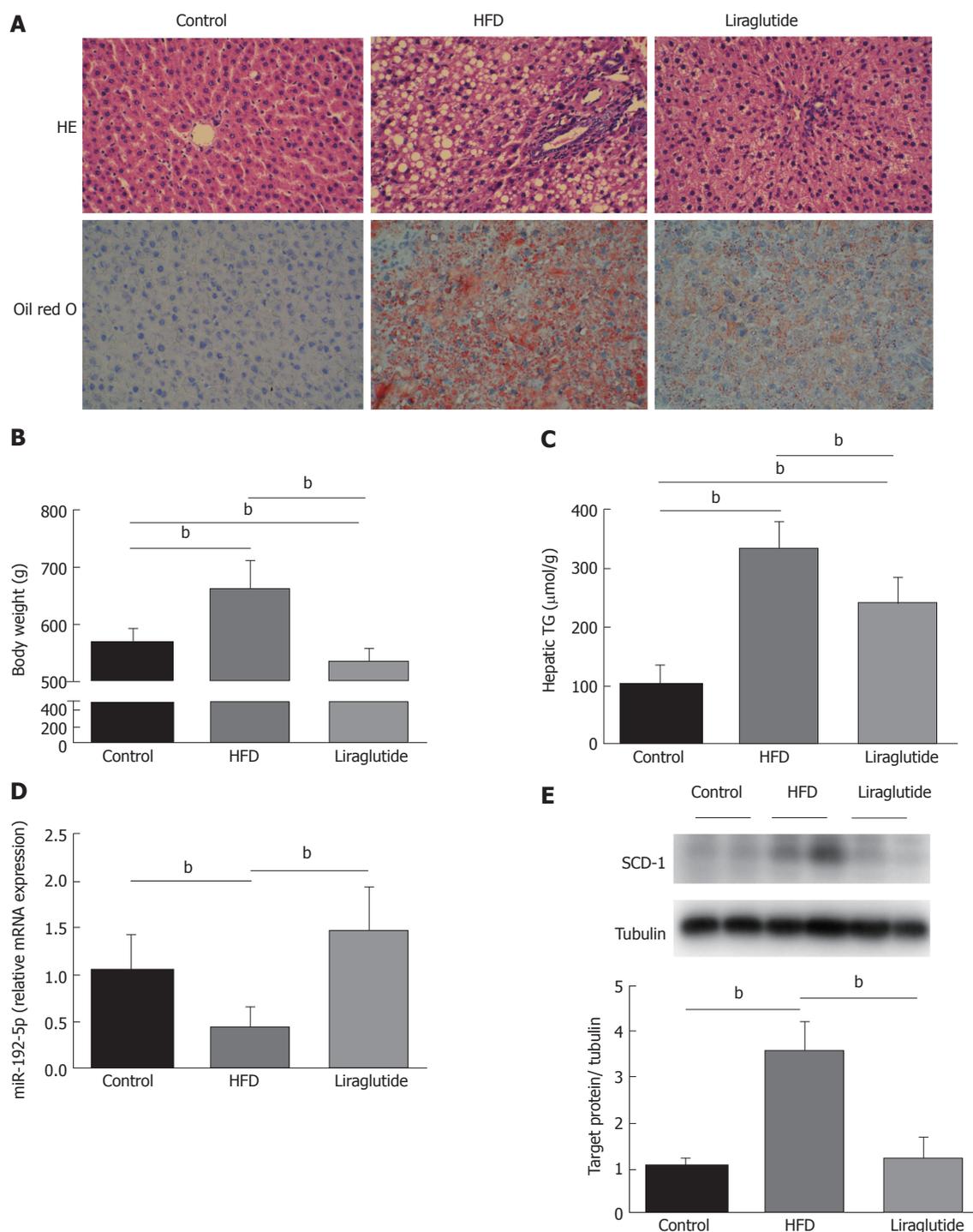


Figure 1 Hepatic miR-192-5p and SCD-1 levels in rat models. H&E staining of livers (A), body weight (B), hepatic TG levels (C), hepatic miR-192-5p levels (D), and SCD-1 protein levels (E) in rats fed an HFD and treated with liraglutide. The data are expressed as the mean ± SEM ($n = 10$ rats/group), ^b $P < 0.01$.

increased to approximately 45 μmol/g after exposure to 0.3 mmol/L PA for 8 h (Figure 2B), and the oil red O staining showed obvious lipid droplets in the cells (Figure 2C). Similar to HFD rat livers, the miR-192-5p levels in PA-treated Huh7 cells showed significant downregulation (55%, $P < 0.01$) compared to the controls (Figure 2D). However, the SCD-1 protein levels showed a 2.64-fold increase in PA-treated Huh7

cells ($P < 0.01$; Figure 2E).

SCD-1 is a direct target of miR-192-5p

Based on the results from the TargetScan, miRanda, and PicTar databases, there are hundreds of predicted targets for miR-192-5p. Among these, SCD-1 is the one that participates in lipid metabolism and increase dramatically in NAFLD. Therefore, we performed dual

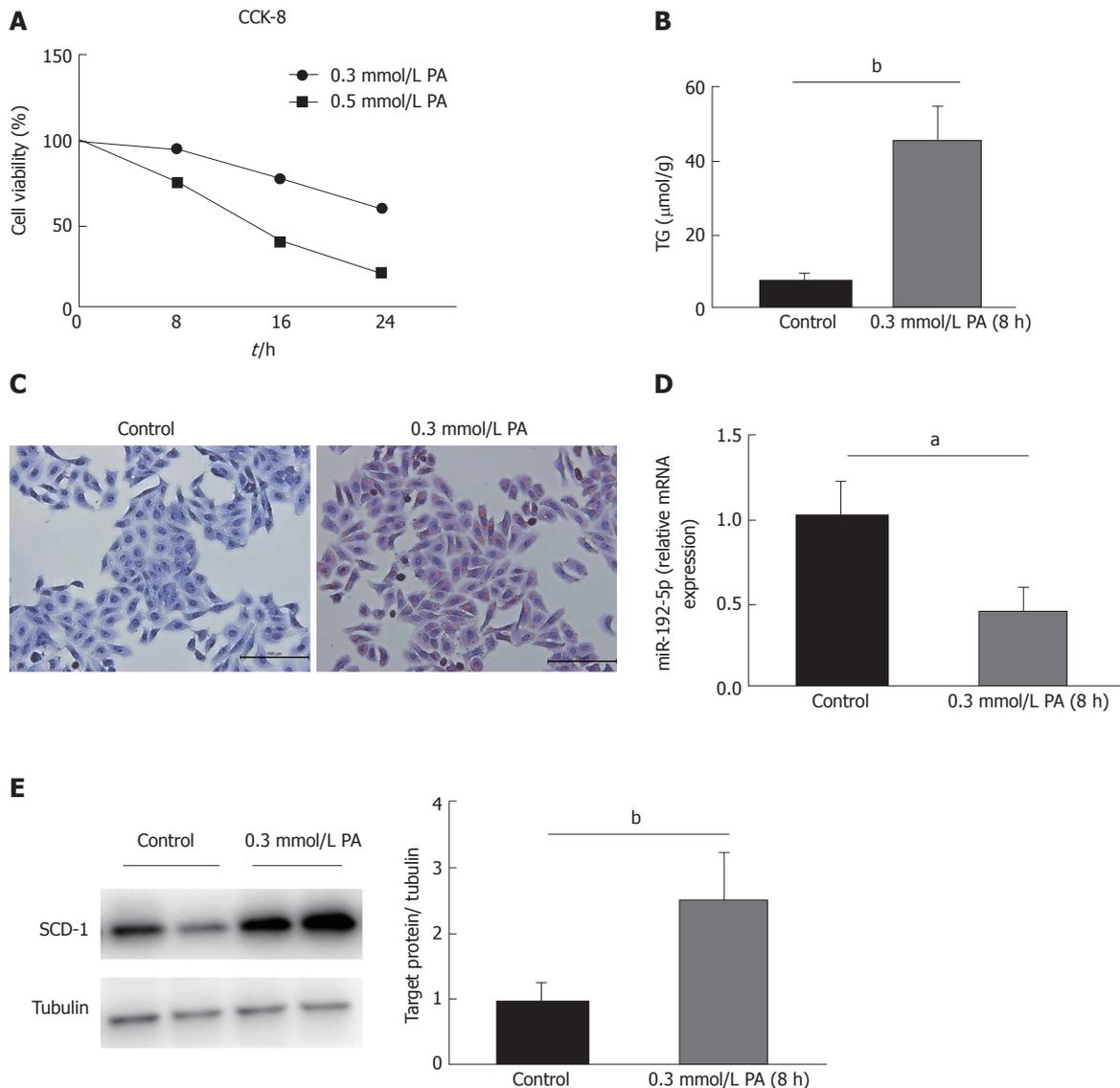


Figure 2 PA induces a decrease in miR-192-5p and an increase in SCD-1 in Huh7 cells. A: The cytotoxic effects of PA on Huh7 cells by the CCK-8 test; B: Cellular TG levels; C: Oil red O staining; D: MiR-192-5p levels; and E: SCD-1 protein levels in Huh7 cells treated with PA. The data are expressed as the mean \pm SEM, $^aP < 0.01$.

luciferase reporter gene assays to validate the direct interaction between miR-192-5p and SCD-1. The binding sites of miR-192-5p with 3' UTR of SCD-1 are shown in Figure 3A, and the miR-192-5p seed sequence was deleted in mutant SCD-1. The luciferase activity in WT SCD-1 3' UTR was suppressed to 60% by miR-192-5p mimic and showed a 1.25-fold increase by miR-192-5p inhibitor compared with their respective negative controls ($P < 0.01$). However, these repressive and stimulatory effects could be abrogated by the mutation of the SCD-1 3' UTR (Figure 3B), suggesting the direct interaction between miR-192-5p and the 3' UTR of SCD-1 mRNA. At the same time, we measured the mRNA levels of SCD-1 in Huh7 cells when transfected with miR-192-5p mimic and inhibitor, but neither of them showed significant differences compared with their respective negative control (Figure 3C). Fortunately, we found an obvious reduction in

SCD-1 protein levels in Huh7 cells transfected with miR-192-5p mimic and an induction of these levels when transfected with miR-192-5p inhibitor compared with their separate negative control ($P < 0.01$; Figure 3D).

miR-192-5p alleviates lipid accumulation by interacting with SCD-1

To evaluate the function of miR-192-5p in lipid metabolism, we transfected Huh7 cells with mimic NC and miR-192-5p mimic and cultured them in 0.3 mM PA medium. From oil red O staining, we found that the overexpression of miR-192-5p could alleviate lipid accumulation in Huh7 cells after exposure to PA for 8 hours (Figure 4A). As expected, Huh7 cells transfected with miR-192-5p mimic resulted in a significant reduction (23%) in cellular TG levels compared with mimic NC after PA exposure ($P < 0.01$; Figure 4B).

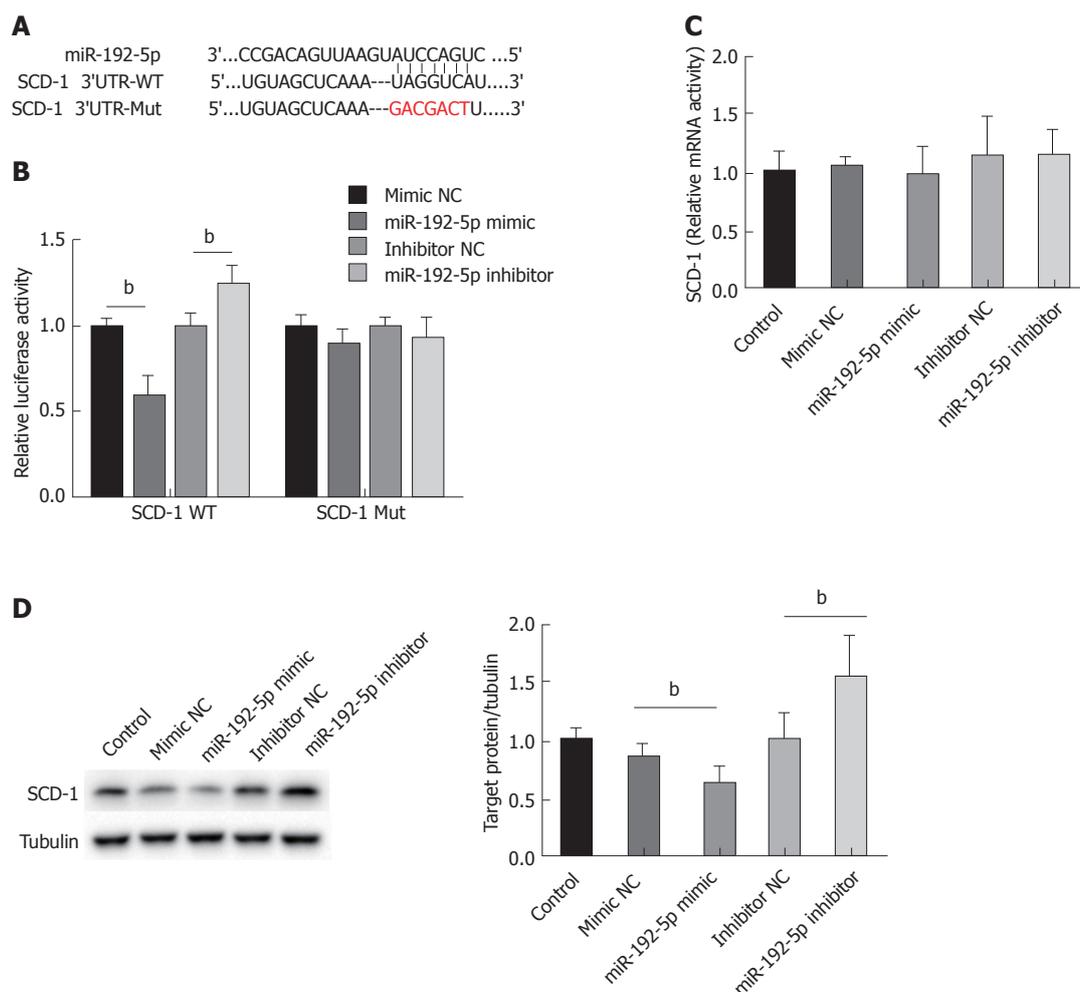


Figure 3 SCD-1 is a direct target of miR-192-5p. A: The binding sites of miR-192-5p with the 3' UTR of SCD-1; B: The dual luciferase activity in the wild type and mutant SCD-1 3' UTRs in 293T cells treated with miR-192-5p mimic and inhibitor, respectively. The mRNA (C) and protein levels (D) of SCD-1 in Huh7 cells transfected with miR-192-5p mimic and inhibitor. The data are expressed as the mean \pm SEM, ^b $P < 0.01$.

We further explored the regulatory effect of miR-192-5p on SCD-1 expression in PA-treated Huh7 cells. The protein levels of SCD-1 were enriched significantly after PA exposure, and the increase could be blocked by the transfection of miR-192-5p mimic (Figure 4C).

SCD-1 mediates the promoting effect of the miR-192-5p inhibitor on lipid accumulation

To further investigate whether the regulation of lipid metabolism by miR-192-5p is mediated by SCD-1, we applied SCD-1 siRNA to perform the functional remedial experiment. As expected, the miR-192-5p inhibitor significantly increased the protein levels of SCD-1 in PA-treated Huh7 cells compared with its negative control, and the concomitant SCD-1 siRNA transfection could abrogate the elevated SCD-1 protein levels (Figure 5A). The inhibition of miR-192-5p could aggravate lipid accumulation in PA-treated Huh7 cells, but this could be reversed by SCD-1 knockdown (Figure 5B). Similarly, cellular TG increased after the inhibition of miR-192-5p compared with its negative control, and this promoting effect could be blocked by the transfection of SCD-1

siRNA ($P < 0.01$; Figure 5C).

DISCUSSION

In the current study, we demonstrated that miR-192-5p decreased in NAFLD both *in vivo* and *in vitro* and the decrease could be reversed after disease remission by liraglutide therapy in animal models. Meanwhile, we confirmed that miR-192-5p had a negative regulatory role in lipid synthesis, which was mediated through its regulation of lipogenetic gene SCD-1.

Because of the abundance of miR-192-5p in the liver, many researchers have focused on miR-192-5p as a serum biomarker of liver injury. For example, serum levels of miR-192-5p have been found to increase in patients with various liver diseases, such as drug-induced liver injury^[18], chronic hepatitis B^[19], hepatocellular carcinoma^[20,21], and NAFLD^[11,22]. Serum miR-122 and miR-192 levels have been reported to differentiate control livers, simple hepatic steatosis, and NASH in clinical studies^[11]. Similarly, our previous research in NAFLD patients also found that miR-122,

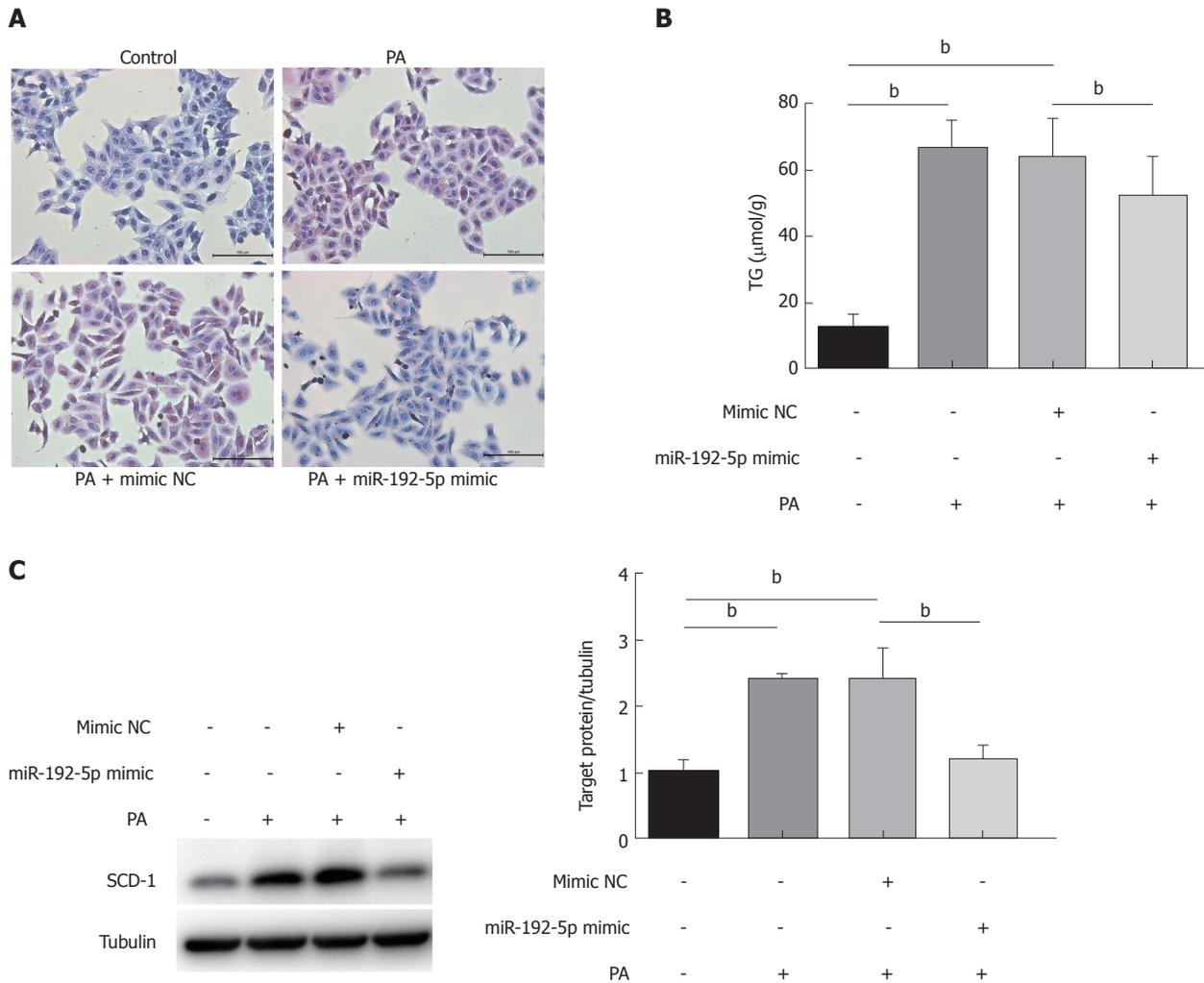
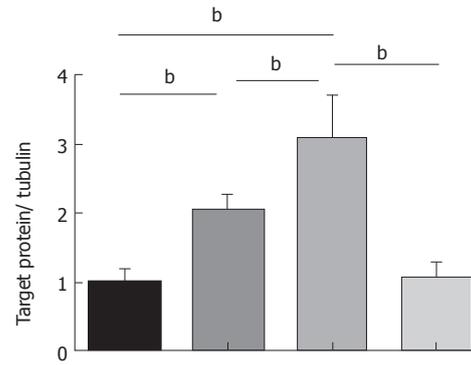
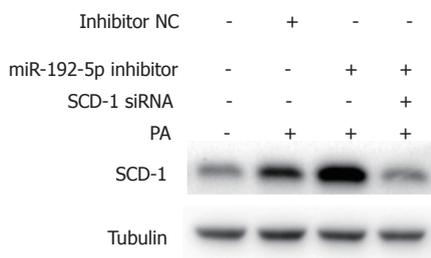


Figure 4 MiR-192-5p alleviates lipid accumulation in PA-treated Huh7 cells. Oil red O staining (A), cellular TG levels (B), and SCD-1 protein levels (C) in Huh7 cells transfected with miR-192-5p mimic and its negative control followed by exposure to PA. The data are expressed as the mean \pm SEM, ^b $P < 0.01$.

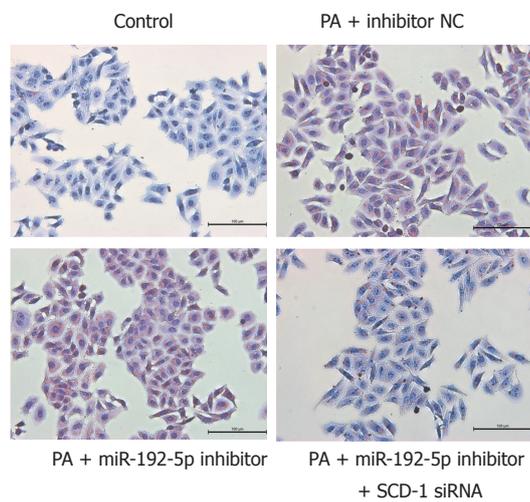
miR-192, and miR-34a showed good correlations with hepatic steatosis and inflammatory activity^[12]. Among the three microRNAs, miR-122 is the most studied in NAFLD and can regulate numerous genes involved in lipid metabolism, such as sterol regulatory element binding protein (SREBP), acetyl coenzyme A carboxylase 2 (ACC2), and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR)^[23,24]. Similarly, miR-34a is characterized as a regulator of peroxisome proliferator-activated receptor α (PPAR- α) and its downstream genes and it induces lipid accumulation in a hepatocyte nuclear factor 4 α (HNF-4 α)-dependent way^[25,26]. However, the regulatory role of miR-192-5p in NAFLD remains unknown. In our *in vivo* and *in vitro* models of NAFLD, miR-192-5p decreased in livers with steatosis and in cells with lipid accumulation. Because liraglutide is a well-known therapeutic option for weight loss and NAFLD^[27], the injection of liraglutide in HFD rats significantly alleviated the steatosis and hepatic TG levels, accompanied by the induction of hepatic miR-192-5p levels. These results may indicate that miR-192-5p has a negative correlation with hepatic lipid accumulation.

Originally, miR-192-5p was identified as an oncogene in some cancers and shows a negative correlation with the liver metastatic potential of colon cancer cells^[28]. The expression of miR-192-5p is regulated partly by transforming growth factor β 1 (TGF β 1) and hepatocyte nuclear factor 4 α (HNF4 α)^[29,30]. A study by Roy *et al* shows that hepatic miR-192-5p can protect against acute liver injury induced by oxidative stress through the mediation of target gene Zeb2, which is a principal transcriptional regulator in cell survival^[13]. Furthermore, docosahexaenoic acid can up-regulate miR-192 levels in enterocyte-like Caco-2 cells, and miR-192 shows a repressive effect on the expression of genes involved in lipid metabolism, such as insulin-like growth factor 1 (IGF1), fatty acid binding protein 3 (FABP3), and very low-density lipoprotein receptor (VLDLR)^[31]. Recently, miR-192-5p was reported to play a role in bisphenol A-triggered NAFLD by regulating SREBF1^[32]. In our NASH animal models fed an HFD and in the Huh7 cells treated with saturated fatty acid PA, which are the most recognized models for this disease, we demonstrated that the hepatic and cellular levels of miR-192-5p decreased significantly and that

A



B



C

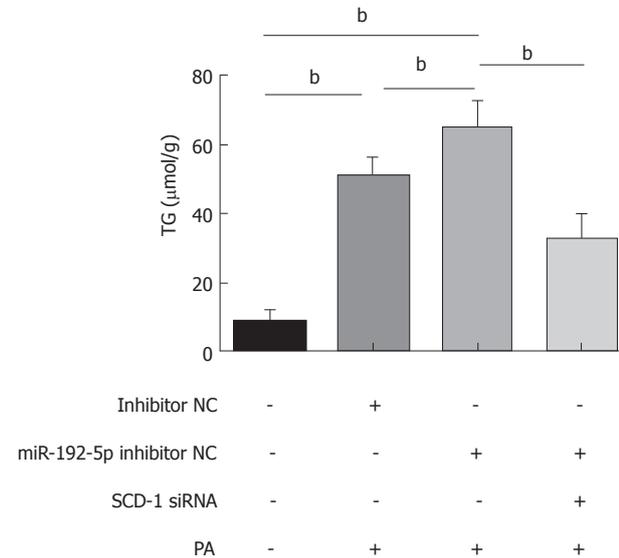


Figure 5 SCD-1 knockdown alleviates the promoting effect of the miR-192-5p inhibitor on lipid accumulation. The protein level of SCD-1 (A), Oil red O staining (B), and cellular TG levels (C) in Huh7 cells transfected with miR-192-5p inhibitor and SCD-1 siRNA followed by exposure to PA. The data are expressed as the mean \pm SEM, ^b*P* < 0.01.

the overexpression of miR-192-5p could alleviate lipid accumulation in Huh7 cells. These results suggested that, in addition to its role in cell death and apoptosis, miR-192-5p could participate in lipid metabolism in NAFLD.

In the livers of NAFLD patients, fat accumulates because of an imbalance between lipid deposition and removal, which is driven by the hepatic synthesis of TG and DNL, especially in conditions of insulin resistance^[16,33]. The contribution of DNL to hepatic TG synthesis and oxidative stress is significant, so inhibitors of DNL, such as Aramchol, are important NAFLD treatments for reducing hepatic fat accumulation^[34,35]. SREBP-1c is reported to be the predominant regulator of DNL in the liver and activates key lipogenic genes such as SCD-1^[17]. Meanwhile, hepatic SCD-1 can also

induce hepatic steatosis independent of upstream regulation by SREBP-1c^[36]. SCD-1 is an enzyme that catalyzes the desaturation of fatty acyl-CoA substrates and participates in the biosynthesis of monounsaturated fatty acids, which constitute the primary components of TG^[37]. Increased SCD-1 activity accelerates the last stage of TG synthesis and stimulates lipid accumulation^[38], but repressed SCD-1 expression up-regulates the components in the insulin-signaling pathway^[39] and reduces obesity in HFD-fed animal models^[40]. Our present study showed an increase in hepatic SCD-1 levels in rats fed an HFD, and treatment with liraglutide could decrease its levels dramatically.

In addition to regulation by upstream transcription factors, SCD-1 may be repressed through a mi-

croRNA-mediated mechanism. Recent studies have demonstrated the roles of miR-125b and miR-29a in the regulation of SCD-1^[41,42]. In our study, we found that a region of the SCD 3' UTR completely matched a seed sequence (5'-UGACCUA-3') at the binding site of miR-192-5p, demonstrating a direct interaction between miR-192-5p and SCD-1. The knockdown and overexpression of miR-192-5p in Huh7 cells showed significant promotion and inhibition effects, respectively, on the protein expression of SCD-1, but no effects were observed on the mRNA levels of this gene. This result suggested that miR-192-5p could regulate the SCD-1 expression at the post-transcriptional level. Since the mRNA targets are only partially complementary to microRNAs in animals, microRNA-induced silencing complexes can mediate post-transcriptional silencing in the absence of mRNA degradation^[43]. In addition, the functional remedial experiment showed that the transfection with SCD-1 siRNA could significantly abrogate the lipid accumulation induced by miR-192-5p inhibitor, indicating that miR-192-5p regulated lipid metabolism at least partly through an interaction with SCD-1.

The potential limitation of our study is that the specific regulatory role of miR-192-5p in NAFLD has not been thoroughly validated *in vivo*. Although one study demonstrated that the overexpression of miR-192-5p could abrogate bisphenol A-induced hepatic steatosis and lipid accumulation in C57BL/6 mice, whether miR-192-5p can alleviate hepatic steatosis in NAFLD patients remains uncertain. Therefore, hepatic miR-192-5p levels and the treatment effect of miR-192-5p need to be validated in clinical practice. It may be difficult to acquire enough participants due to the invasive method required for the retrieval of liver samples from NAFLD patients, but it would be of practical implications if clinical experiments support our claims going forward. In addition, as the therapy with liraglutide in NAFLD is accompanied by the upregulation of hepatic miR-192-5p levels, the rationale between liraglutide and hepatic miR-192-5p is also an open question.

In conclusion, we demonstrated that hepatic miR-192-5p levels decreased in NASH rat models fed an HFD and that this decrease could be reversed after disease remission by liraglutide therapy. We first confirmed that miR-192-5p had a direct regulatory effect on lipogenic gene SCD-1 and that this mediated the negative regulatory effect of miR-192-5p in lipid synthesis in NAFLD. These data complement the regulators involved in the progression of NAFLD and help us better understand the role of epigenetic factors in this disease. Meanwhile, our study suggests that the overexpression of miR-192-5p may reflect a

promising treatment strategy for NAFLD, which calls for more validated data in the future.

ARTICLE HIGHLIGHTS

Research background

Based on the "multiple hit" theory of non-alcoholic fatty liver disease (NAFLD) pathogenesis, lipid accumulation initiates simple hepatic steatosis and subsequently triggers multiple insults, ultimately inducing non-alcoholic steatohepatitis, cirrhosis, and even hepatocellular carcinoma. In addition to the classical factors involved in the progression of NAFLD, microRNAs, which represent epigenetic alterations, have been identified as important post-transcriptional regulators in the pathogenesis of this disease.

Research motivation

Due to the abundance of miR-192-5p in the liver, many researchers have focused on miR-192-5p as a serum biomarker of liver injury, such as drug-induced liver injury, chronic hepatitis B, hepatocellular carcinoma, and NAFLD. Because microRNAs are important regulators in a wide spectrum of biological processes and metabolic homeostasis, the role of microRNAs in NAFLD pathogenesis is of particular interest. Our previous research in NAFLD patients found that serum miR-192-5p levels could differentiate different stages of NAFLD and showed good correlations with hepatic steatosis and inflammatory activity, but little is known about its regulatory role in lipid metabolism.

Research objectives

We aimed to evaluate the hepatic levels of miR-192-5p in rat models of NAFLD, and figure out the role of miR-192-5p in lipid metabolism. After the present study, we have identified the lipogenic gene SCD-1 as a target gene of miR-192-5p, confirming the negative regulatory effect of miR-192-5p in lipid synthesis in NAFLD. These realized objectives give us a better understanding about the functional mechanism of miR-192-5p in NAFLD and provide a new insight in the miRNA-intervention treatment strategy for this disease.

Research methods

We conducted this study in HFD-fed rats and palmitic acid-treated Huh7 cells. The hepatic and hepatocellular levels of miR-192-5p in NAFLD were evaluated using quantitative real-time polymerase chain reaction both *in vivo* and *in vitro*. The SCD-1 protein levels were examined by Western blot analysis. Oil red O staining and TG assay were used to detect the lipid accumulation in rat livers and hepatocytes. Overexpression and knockdown of miR-192-5p were performed in Huh7 cells with miR-192-5p mimic and inhibitor. Luciferase reporter assays confirmed the direct interaction between miR-192-5p and SCD-1.

Research results

In the current study, we found that miR-192-5p decreased in NAFLD both *in vivo* and *in vitro* and the decrease could be reversed after disease remission by liraglutide therapy in animal models. Meanwhile, we confirmed that miR-192-5p had a negative regulatory role in lipid synthesis, which was mediated through its regulation on lipogenic gene SCD-1. However, the specific regulatory role of miR-192-5p in NAFLD patients remains unclear, which needs to be validated in clinical studies.

Research conclusions

The authors demonstrated that miR-192-5p decreased in NAFLD conditions both *in vivo* and *in vitro*, which could provide more evidence for the clinical use of circulating miR-192-5p as a biomarker in NAFLD. We first confirmed that miR-192-5p showed direct regulation on lipogenic gene SCD-1 and that this could mediate the negative regulatory effect of miR-192-5p in lipid synthesis in NAFLD. These data complement the regulators involved in the progression of NAFLD and help us better understand the role of epigenetic factors in this

disease. Meanwhile, the study suggests that the overexpression of miR-192-5p may reflect a promising treatment strategy for NAFLD, which calls for more validated data in the future.

Research perspectives

This study suggests that the miRNA intervention may be a promising treatment strategy for NAFLD. The future study might focus on the specific regulatory role of miR-192-5p in NAFLD *in vivo*, and the therapeutic effect of miR-192-5p needs to be validated in clinical practice. It would be of practical implications if clinical experiments support our claims going forward.

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