Inhibition of miR-499-5p expression improves nonalcoholic fatty liver disease

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is one of the leading causes of chronic liver diseases. However, the pathogenesis of NAFLD is largely unknown. Here, we investigated the specific role of miR-499-5p in NAFLD. Method: Free fatty acid (FFA) was used to induce HL-7702 cell line to establish a NAFLD cell model, and animal models of NAFLD were constructed by feeding C57BL/6 mice with high fat diet (HFD). Expression levels of miR-499-5p in the HL-7702 cells and C57BL/6 mice were determined by RT-qPCR. In addition, functional experiments were carried out through transfecting miR-499-5p inhibitor into NAFLD cells, and injecting NAFLD mice with a lentiviral vector with knock down the miR-499-5p. Furthermore, the effects of miR-499-5p inhibition on lipidation and inflammation were investigated by oil red O staining, HE staining, and biochemical analysis. Results: Compared with normal controls, the expression of miR-499-5p was significantly up-regulated in NAFLD cells and tissues in mouse (P < 0.05). After NAFLD cells transfected by miR-499-5p inhibitor, the expression of miR-499-5p was inhibited, the lipid deposition and content of TG were reduced, and the lipidation was improved (P < 0.05). Simultaneously, after NAFLD mice were injected with knocked down the miR-499-5p lentiviral vector, the degree of lipid droplet deposition and content of TG were also reduced. Besides, it also decreased the levels of TC and AST in serum, and improved hepatic lipid metabolism (P < 0.05). Conclusion: Inhibition of miR-499-5p expression improved NAFLD in mice, which provided a new direction for the treatment of NAFLD.

Background

Non-alcoholic fatty liver disease (NAFLD) is a disease associated with abnormal lipid accumulation in hepatocytes, which might develop into fibrosis, cirrhosis and even canceration under certain circumstances [1, 2]. Steatosis (accumulation of fat droplets in hepatocytes), inflammation, balloon damage, fibrosis and cirrhosis are the pathological features of NAFLD [3]. Causes of NAFLD mainly include changes in fat metabolism, proinflammatory and anti-inflammatory molecular imbalances [4]. Generally, 30% of the American population are affected by NAFLD [5], and the prevalence of NAFLD in Asia is about 25% [6]. In fact, NAFLD is a liver manifestation of metabolic and inflammatory liver disease, a high fat (HF) diet triggers steatosis and induces lipotoxicity, inflammation and insulin resistance [7]. Correspondingly, the traditional treatments for NAFLD focuses on lifestyle interventions (control diet and exercise) [9, 10] and drug therapy[8, 9]. With the widespread outbreak of NAFLD, molecular targeting technology has also become a hot spot for the treatment of this disease.

MiRNAs are a class of non-coding RNAs found in eukaryotes with regulatory functions, circulate freely in human plasma, and closely related to different pathology including NAFLD. For instance, up-regulation of miR-181b alters lipid metabolism in patients with NAFLD, while inhibition of miR-181b expression reduces hepatic steatosis [10]. Moreover, the levels of miR-34a and miR-122 in the serum of patients with NAFLD are higher than those in the normal population, which could be used as a potential biomarker for distinguishing between NAFLD patients and healthy people [11]. MiR-499-5p, is a biomarker of diabetes mellitus with the risk and prognosis of diabetes mellitus [12]. NAFLD could cause impaired glucose
tolerance, leading to type 2 diabetes [13]. Additionally, elevated levels of miR-499-5p could participate in innate immune responses and viral replication, whereas decreased miR-499-5p in early fibrosis may mediate chronic hepatitis [11]. Although the expression of miR-499-5p was closely related to the pathology of NAFLD-related diseases, the regulation mechanism of miR-499-5p in NAFLD was still unclear.

In this study, models of NAFLD were established based on C57BL/6 mice and HL-7702 cells, and then the quantitative real-time polymerase chain reaction (qRT-PCR) analysis, HE staining, Oil red O staining, as well as GPO Trinder enzymatic assay were investigated. Our findings suggest that miR-499-5p may represent a novel indicator of fat metabolism in NAFLD and may be a potential target for diagnosis and therapy.

Methods

Construction of NAFLD cell model

Human normal liver cells HL-7702 cells (Shanghai Institute of cell biology, Chinese Academy of Sciences) were seeded in 6-well plates at 5 x 10^5 cells/well. They were divided into control group and NAFLD model group (FFA group). After the adherent cells growth, cells in the control group were added with RPMI1640 medium containing 1% bovine serum albumin (BSA), and cells in the FFA group were added with high fat medium containing 1 mmol/L free fatty acid (FFA). Subsequently, cells were cultured in a 37 °C incubator containing 5% CO_2. After induction of high-fat culture solution for 24 h, the cells were subjected to oil red O staining and triglyceride determination, respectively.

Cell transfection

HL-7702 cells were divided into control group (normal liver cells), FFA group (NAFLD cells), FFA + NC group (NARLD cells transfected with miR-499-5p negative-control inhibitor) and FFA + miR-499-5p inhibitor group (NAFLD cells transfected with miR-499-5p inhibitor). The control group was cultured in the usual medium, while the other three groups were cultured in the FFA high-fat medium. The normal liver cells and the successfully modeled HL-7702 cells were first collected, resuspended and counted, and then inoculated into a 24-well plate in an equal amount, and cultured in a 37°C and 5% CO_2 incubator. When the cells were confluent to 60-80%, siRNA was transfected by liposome Lipofectamine™ 2000, respectively. Next, 3 μL Lipofectamine™ 2000 and 1 μL siRNA was diluted by 50 μL Opti-MEM® Reduced Serum Medium, and the liquid was kept at room temperature for 5 min after dilution. Then, the diluted siRNA and Lipofectamine™ 2000 were gently mixed and incubated for 20 min at room temperature. Finally, miR-499-5p inhibitor and Lipofectamine™ 2000 complex were added to each well, and the plates were gently shaken and incubated at 37 °C in a 5% CO_2 incubator for 24 h.

Construction and grouping of NAFLD mouse models
Twenty-four SPF male C57BL/6 mice of 4 weeks old, weighing 18-20 g, were purchased from the Experimental Animal Center of Zhejiang Academy of Medical Sciences (Zhenjiang, China). Ordinary feed contained 8% rice bran, 51% corn, 30% soy flour, 3% bone meal, 1.3% multivitamin and 6.7% mineral, and high fat diet contained 80.5% basic feed, 2% cholesterol, 7% lard, 10% egg yolk powder and 0.5% bile salt.

After 7 days of adaptive feeding, 24 mice were randomly divided into SCD group and HFD group. Mice in SCD group (n = 8) were fed with basal diet, and mice in NAFLD model (HFD group) (n = 16) were fed with high fat diet. In addition, feeding environment conditions were a temperature range of 20-22 °C, a humidity range of 50-55%, and a brightness of 12 hours each. The experimental animals were free to eat and drink, and 4 was kept in one cage. After feeding for four weeks, 4 mice in the control group and the model group were sacrificed to test whether the NAFLD model was successfully constructed. Then, the NAFLD model group (n = 12) was subdivided into HFD group, HFD + NC group and HFD + miR-499-5p inhibitor group. The treatment was as follows: mice in the HFD group (n = 4) were injected with 100 μL physiological saline in the tail vein; lentiviral expression vector containing 100 mL unrelated sequence (viral quantity was 2 \( \times 10^7 \) TU) was injected into caudal vein of mice in HFD + NC group (n = 4), and mice in the HFD + miR-499-5p inhibitor group (n = 4) were injected with a lentiviral expression vector containing 100 mL inhibitory sequence in the tail vein (viral quantity was 2 \( \times 10^7 \) TU). Then, the high fat diet was continued for 4 weeks, and all mice were killed by decollation after 8 weeks. Before sacrificed, the mice were fasted overnight, the right eyeballs were removed and about 1.5 ml blood was taken for biochemical detection. Next, the middle left lobe of the liver was treated with HE staining for observation of pathological changes in the liver. Additionally, the remaining liver tissue was washed with PBS and preserved in liquid nitrogen. All animal experiments followed the guidelines for the management and use of laboratory animals.

**Measurement of total cholesterol (TC) and aspartate aminotransferase (AST)**

After feeding for 8 weeks, mice were fasted overnight and anesthetized by intraperitoneal injection of sodium pentobarbital (0.05 mg/g body weight). The eyeballs were removed and blood was collected with a 1.5 mL EP tube. The blood was centrifuged at 3000 rpm for 15 min at 4 °C to separate the serum. Finally, the levels of TC and AST in serum were measured using a Cobas8000 automatic biochemical analyzer (Roche, USA). After the blood collection, mice were decapitated and the liver was harvested.

**qRT-PCR**

Total RNA of normal liver cells HL-7702 and NAFLD cells was extracted by TRIZOL reagent (Thermo Fisher scientific, New York, USA). Simultaneously, total RNA of normal mouse liver tissues and liver tissues of NAFLD mice were extracted by RNAprep pure Tissue Kit (TIANGEN biotech Co., Ltd., Beijing, China). First, 1 μg total RNA was used as the initial template, and the total reaction system was 20 μL. The reverse transcription synthesis cDNA was performed on the Gene Amp PCR System 9700 using the FastQuant cDNA First Chain Synthesis Kit (FastQuant RT Kit (with gDNase), KR 106, TIANGEN, China). Subsequently, qRT-PCR was performed on a Rotor-Gene 3000 Real-time PCR instruments (Corbett
Research) using a SuperReal Fluorescence Quantitative Premix Kit (SuperReal PreMix Plus, FP205, TIANGEN, China) and taking 1 μL cDNA as a template. The reaction conditions were pre-denaturation at 95 °C for 15 min, denaturation at 95 °C for 15 s, annealing at 60 °C for 60 s for 40 cycles, and U6 (F: 5’-ATTGGAACGATACAGAGAAGATT-3’; R: 5’-GGAACGCTTCACGAATT TG-3’) as an internal reference for miR-499-5p (F: 5’-ACTGCTTAAGACTTGGAGTGA-3’; R: 5’-TACATTGGTGTCGTGGAGTCGCAA-3’). The relative expression of miR-499-5p was calculated using the $2^{-\Delta\Delta Ct}$ formula. The primers used in the experiment were synthesized by Invitrogen Company.

**HE staining**

Four mice were taken from each group, and they were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 min. Next, thoracic liver tissue was taken and the blood was washed away by PBS. Then, liver tissue was paraffin-embedded, sectioned and stained with HE staining. The HE staining procedure was as follows: the sections were dewaxed by xylene and dehydrated by alcohol gradient, then stained with hematoxylin stain for 1 min, soaked in PBS for 1 min, rinsed with pure water until the sections were fully blue. Next, the sections were stained with eosin solution for 1 min, and then placed in gradient alcohol and xylene for dehydration and transparency. Finally, the neutral resin was mounted and the histopathological changes were observed under ordinary light microscope.

**oil red O staining**

The dry powder of 0.25 g oil red O was dissolved in isopropanol to 50 mL and stored at 4 °C in the dark. Before the cells and liver tissue were stained, 4 mL Oil Red O stock solution was diluted with 6 mL pure water to form Oil Red O staining solution. Immediately, the cell culture was removed and the cells were fixed with 10% neutral formaldehyde for 15 min. Then, the cells were stained with oil red O staining solution for 10 min and counterstained with hematoxylin for 5 min. After rinsing with ddH$_2$O, the cells were observed and photographed under the microscope.

The liver tissue stored in liquid nitrogen was taken out and cut into 5 μm thick at -34 °C. Then, the step of staining the liver tissue with Oil Red O staining solution was the same as the staining of the cells.

**Determination of TG**

TG was mainly determined by the GPO Trinder enzymatic method. The cell culture medium was aspirated, and 200 μL lysate was added to each well of a 6-well plate to lyse the cells. Next, the standard glycerin and cell lysate were mixed with the working solution, respectively, and the mixture was allowed to stand at 37 °C for 10 min. The TG concentration of the cells was measured at 570 nm using a microplate reader (Model 680, BIO-RAD, USA). Additionally, to detect the content of TG in the liver tissue of mice, 50 mg liver tissue was weighed, and then 1 mL of the lysate was added and ground into a uniform mixture. The remaining detection steps for the content of TG in mouse liver tissue were the same as procedure for cells.
**Statistical analysis**

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean ± standard deviation (M ± SD). Statistical analysis was carried out using Student’s t-test between two groups. All experiments were repeated at least three times. P < 0.05 indicated that the difference was statistically significant.

**Results**

**NAFLD cell and mouse model were successfully constructed**

To determine whether NAFLD cell model was successfully constructed, we performed Oil Red O staining. The results revealed that cells in the control group were characterized by clear cell edges, abundant cytoplasm, intact nuclear membrane, large nucleus, and visible mitotic phase, and a little small red-stained lipid droplet (Figure 1A). In the FFA group, the cells became round and the nucleus was large, and some of the nucleus was squeezed to one side, and a large amount of red-stained lipid droplets were observed in the cytoplasm (Figure 1A). In addition, the content of intracellular TG was obviously increased in the FFA group compared with the control group (P < 0.05, Figure 1B). The results indicated that the NAFLD cell model was successfully established by culturing with FFA high fat culture medium for 24 h.

Oil red O staining showed that a large number of red-stained lipid droplets were observed in the cytoplasm of hepatocytes in the HFD group (Figure 2A). HE staining showed that the hepatic lobules in the SCD group were clear, the liver cells cable was neat and orderly, the liver sinus was normal, the liver cells had no obvious lesions, and the nuclear structure was clear. In HFD group, diffuse hepatic steatosis was occurred in the liver of mice, which showed microvesicular steatosis, and no obvious inflammatory cell infiltration, hepatocyte necrosis and fibrosis (Figure 2B). Simultaneously, compared with the control group, the content of TG in liver of the HFD group was observably increased (P < 0.05, Figure 2C). The results suggested that the NAFLD model was successfully established after 4 weeks of HFD feeding.

**miR-499-5p was highly expressed in NAFLD cell model and mouse model**

The expression level of *miR-499-5p* in the FFA group was significantly higher than that in the control group (P < 0.05, Figure 3A). Meanwhile, the level of *miR-499-5p* in HFD group was distinctly increased than that in the SCD group (P < 0.05, Figure 3B).

The expression of *miR-499-5p* in HL-7702 cells was shown in Figure 4A. Compared with the control group, the expression levels of *miR-499-5p* in FFA, FFA + NC, FFA + *miR-499-5p* inhibitor groups were higher (P < 0.05), while the expression levels of *miR-499-5p* in FFA + *miR-499-5p* inhibitor group were lower than those in FFA and FFA + NC groups (P < 0.05). There was no significant difference between FFA and FFA + NC groups (P > 0.05). Figure 4B was a representation of the level of *miR-499-5p* in mouse liver tissue. Compared with the control group, the expression levels of *miR-499-5p* in HFD, HFD + NC, HFD + *miR-499-5p* inhibitor groups were higher (P < 0.05), while the expression levels of *miR-499-5p* in HFD + *miR-499-5p*
inhibitor group were lower than those in HFD and HFD + NC group (P < 0.05). There was no significant difference between HFD and HFD + NC groups (P > 0.05).

Inhibition of miR-499-5p expression attenuated hydroxy steatosis in HL-7702 cells

Hepatic steatosis is commonly observed in histopathological evaluation of patients with NAFLD. Also, it is a hallmark of NAFLD, which is defined as a high intrahepatic glycerol (TG) content [14]. In this study, we investigated the effect of miR-499-5p on hydroxy steatosis, after oil red O staining, we found that a large number of red-stained lipid droplets appeared in the cytoplasm of FFA, FFA + NC and FFA + miR-499-5p inhibitor groups. Compared with the FFA and FFA + NC groups, the lipid droplets in the cytoplasm of the FFA + miR-499-5p inhibitor group were significantly reduced (Figure 5A). GPO Trinder assay showed that the content of TG in the FFA + miR-499-5p inhibitor group was observably lower than that in the FFA and FFA + NC groups (P < 0.05, Figure 5B).

Inhibition of miR-499-5p expression mitigated liver cell steatosis in mice

After feeding with HFD for 8 weeks, diffuse hepatocyte steatosis was found in the liver of HFD and HFD + NC mice, and the degree of steatosis was visibly worse than that of 4 weeks. Moreover, there was mixed steatosis predominantly vesicular without obvious inflammatory cell infiltration, hepatocyte necrosis and fibrosis. The degree of steatosis of the liver tissue of the miR-499-5p inhibitor group was markedly improved, and there were no other histological manifestations such as inflammatory cell infiltration, hepatocyte necrosis and fibrosis in comparison with the HFD + NC group (Figure 6A). Additionally, as shown in Oil red O staining, the degree of lipid droplet deposition in the cytoplasm of liver tissue cells of HFD and HFD + NC group was almost the same. The mice treated with miR-499-5p inhibitor showed clearly reduced lipid droplet deposition in the cytoplasm of liver tissue cells when compared with corresponding control groups (Figure 6B). GPO Trinder assay showed that the difference content of TC between HFD and HFD + NC group was not significant (P > 0.05). Compared with the HFD and HFD + NC groups, the contents of TG in the liver tissue of the HFD + miR-499-5p inhibitor group was significantly decreased (P < 0.05, Figure 6C).

Inhibition of miR-499-5p expression improved liver damage

High AST level is an indicator of moderate-to-severe fibrosis in NAFLD patients [15]. Moreover, high TC levels are associated with a greater risk of NAFLD [16]. Compared with SCD group, the levels of TC and AST in serum of mice in HFD, HFD + NC and HFD + miR-499-5p inhibitor groups were evidently higher (P < 0.05), while in miR-499-5p inhibitor group they were markedly decreased than those in HFD and HFD + NC groups (P < 0.05, Figure 7).

Discussion

Recently, miRNAs have been found to play important roles in several animal models of NAFLD [17]. Deregulated miRNAs are involved in the transition from hepatic steatosis to steatohepatitis in rat model.
of NAFLD [18]. In this study, qRT-PCR study showed that the expression of \textit{miR-499-5p} increased in NAFLD model group compared with the control group and \textit{miR-499-5p} inhibitor could alleviate the steatosis of liver cells with NAFLD, reduce the content of TC and AST in the serum, which suggested that reduced \textit{miR-499-5p} levels contribute to alleviate the steatosis and improve the liver damage of mice. \textit{MiR-499-5p} might become a new target for the treatment of NAFLD.

miRNAs have been found as useful serum biomarkers in the diagnosis and treatment of various diseases [19]. Previous study has reported that serum levels of miR-122 were correlated with severity of liver steatosis, and may be a useful screening biomarker for NAFLD [20]. In addition, increased miR-34a has been described in circulating serum of NAFLD patients [26]. MiR-21 could regulate triglyceride and cholesterol metabolism by inhibiting the expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the serum of patients with NAFLD [21]. Ding \textit{et al.} [22] have reported that miR-34a inhibitors counteract NAFLD by inhibiting lipid accumulation. In this study, the expression of \textit{miR-499-5p} increased in NAFLD model group and inhibition of \textit{miR-499-5p} expression attenuated liver cell steatosis, suggesting that \textit{miR-499-5p} may be suited to assess early NAFLD and could serve as a tool for understanding the progression of NAFLD.

Based on our results, reducing the accumulation of TG content can alleviate the steatosis of NAFLD. Similar to our findings, Das \textit{et al}, have found that miR-30c-5p targeting regulates fatty acid synthase, reduces triglyceride accumulation and lipid deposition, and improves NAFLD [23]. In fact, abnormal accumulation of TG in the liver is one of the characteristics of NAFLD [24, 25]. Studies reveal that a large amount of lipid deposition is observed in the cell model of NAFLD, and the levels of TG are elevated [26, 27]. Besides, when lipid accumulation in the liver cells of NAFLD mice is increased, the TG content in liver tissue is also increased [28]. Consistent with previous studies, we found that the \textit{miR-499-5p} inhibitor was also found to be clearly reduced the TG content of the NAFLD models, suggesting that \textit{miR-499-5p} might improve the fatty degeneration of NAFLD hepatocytes. In another study, Gao \textit{et al.} [29] point out that atorvastatin has become a strategy for the treatment of NAFLD by promoting fat solubilization and reducing the accumulation of TG in the liver.

In addition to the mechanisms mentioned above, the expression of genes involved in lipid metabolism is regulated by affecting the methylation modification of adipose DNA to reduce the content of TG and TC, and thus liver damage caused by NAFLD is effectively alleviated [30]. Recent research has indicated that compared with non-NAFLD population, the levels of TC in NAFLD patients increase evidently [31, 32]. Moreover, as NAFLD aggravated, the content of TC is evidently increased [33]. OU \textit{et al.} [34] have found that the levels of TC and AST in patients with NAFLD are obviously higher than those in healthy subjects. Naturally, the levels of TC and AST in the HFD group are significantly elevated [35, 36]. In this study, we also found that inhibition of \textit{miR-499-5p} expression reduced serum TC and AST levels in NAFLD mice, suggesting that decrease of \textit{miR-499-5p} level might ameliorate liver damage caused by NAFLD. In agreement with our results, Zhang \textit{et al.} [37] improved liver function in rats with NAFLD by reducing serum TC and AST levels, and achieved the effect of alleviating NAFLD induced by HFD. Dong \textit{et al.} [38]
reduced the serum AST and TC levels in NAFLD mice by increasing the expression of PPARγ mRNA in the liver, and thus acting as a therapeutic agent for NAFLD.

Conclusions

In conclusion, we have shown that decreased expression of miR-499-5p in mice models of NAFLD likely plays a critical homeostatic role to prevent excessive lipid accumulation in livers which could ultimately give rise to liver damage. Future studies determining how miR-499-5p interacts with lipid, fibrosis and inflammatory pathways in NAFLD are needed and could offer new insights into the pathogenesis of NAFLD.

Abbreviations

Nonalcoholic fatty liver disease (NAFLD)
Free fatty acid (FFA)
high fat diet (HFD)
high fat (HF)
quantitative real-time polymerase chain reaction (qRT-PCR)
bovine serum albumin (BSA)
total cholesterol (TC)
aspartate aminotransferase (AST)
intrahepatic glycerol (TG)

Declarations

Ethnics approval and consent to participate

This study was conducted after obtaining Medical College of Qingdao University ‘s approval and written informed consent from the patients.

Consent for Publication

All contributing authors have agreed to submit this manuscript and all authors approved to publish this study.

Availability of data and materials
All data from the hospital.

**Competing interests**

The Authors declare that they have no conflicts of interest to disclose.

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**Authors’ Contributions**

HYL and TW: conception and design and analysis of data, drafting the article. XC, SYX and JJ: drafting the article. NHS, RL and YNX: revising the article critically for important intellectual content. All authors have read and approved the manuscript.

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Figures

Figure 1

Successful construction of NAFLD cells model (A) Lipid droplet deposition in control group and FFA group (oil red O staining, 400×), (B) The content of TG in control group and FFA group. Versus control group, *P < 0.05. Bar = 100 μm

Figure 2

Successful construction of NAFLD mouse model (A) Lipid deposition in mouse hepatocytes (oil red O staining, 400×), (B) Performance of liver pathology in mice (HE staining, 400×), (C) The content of TG in mouse liver tissue, *P < 0.05, versus SCD group. Bar = 50 μm
Figure 3

Expression of miR-499-5p in NAFLD cell and mouse models (A) Expression of miR-499-5p in normal hepatocytes and NAFLD cell model was detected by qRT-PCR, (B) Expression of miR-499-5p in normal mice and NAFLD model mice was measured by qRT-PCR. *P < 0.05, versus control group and SCD group, respectively.
Figure 4

Successful transfection of miR-499-5p inhibitor (A) miR-499-5p inhibitor was successfully transfected into HL-7702 cells, #P < 0.05, versus control group, *P < 0.05, versus FFA and FFA + NC group. (B) MiR-499-5p inhibitor was successfully transfected in mice. #P < 0.05 versus control group, *P < 0.05, versus HFD and HFD + NC group.

Figure 5

Inhibition of miR-499-5p expression attenuated steatosis in HL-7702 cells (A) Changes in the degree of lipidation of HL-7702 cells after transfection with miR-499-5p inhibitor, (B) Changes in the content of TG after transfection with miR-499-5p inhibitor. *P < 0.05, versus FFA and FFA + NC groups. Bar = 100 μm

Figure 6
Down-regulating the expression of miR-499-5p mitigated liver steatosis in mice with NAFLD. (A) Liver pathology of mice (HE staining, 400×), (B) Lipid deposition in mouse liver tissue (oil red O staining, 400×), (C) The contents of TG in mouse liver tissue, *P < 0.05, versus HFD and HFD + NC group. Bar = 50 μm

**Figure 7**

Down-regulating the expression of miR-499-5p increased TC and AST levels in mouse with NAFLD. (A) Cobas8000 automatic biochemical analyzer was used to detect the content of TC, (B) The content of AST in mouse serum was detected by Cobas8000 automatic biochemical analyzer. *P < 0.05 versus SCD group, #P < 0.05, versus HFD and HFD + NC group

**Supplementary Files**

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