MiR-18 regulates lipid metabolism of non-alcoholic fatty liver disease via IGF1

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Abstract

We aimed to illustrate the regulatory effect of miR-18 on the onset of non-alcoholic fatty liver disease (NAFLD). MiR-18 level in liver tissues collected from NAFLD patients and mice was detected. In vivo and in vitro influences of miR-18 on biochemical indexes, glucose tolerance and insulin resistance (IR) in NAFLD were determined. H&E staining was conducted to observe hepatic steatosis in NAFLD mice. The downstream target of miR-18 was finally detected by luciferase assay. MiR-18 was upregulated in liver tissues collected from NAFLD patients and mice. Knockdown of miR-18 reduced levels of AST, ALT, TG and TC in NAFLD mice and culture medium of FFA-induced LO2 cells. Meanwhile, knockdown of miR-18 alleviated hepatic steatosis and IR in NAFLD mice. IGF1 was the target of miR-18, and it was negatively regulated by miR-18. MiR-18 is upregulated in NAFLD patients and mice. Knockdown of miR-18 alleviates HFD-induced hepatic steatosis and IR through interacting with IGF1 to regulate to lipid metabolism and insulin signals.

Keywords: NAFLD, MiR-18, IGF1, Lipid metabolism.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a prevalent metabolic liver injury intricately associated with insulin resistance (IR) and genetic predisposition. Diverging from the narrative of excessive alcohol consumption, NAFLD manifests pathological alterations akin to alcoholic liver disease. This condition is classified into non-alcoholic simple fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) [1,2], with its incidence on the rise in recent years. Factors such as economic development, shifts in lifestyle, and the consumption of high-fat, high-calorie diets have contributed to the escalating prevalence. Noteworthy risk factors for NAFLD encompass a high body mass index (BMI), diabetes, IR, and metabolic syndromes. Currently, NAFLD affects approximately 25% of the global adult population [3], underscoring the urgent need for the development of effective prevention and treatment strategies.

Despite its widespread prevalence, the pathogenesis of NAFLD remains largely elusive. The disease induces alterations in histone modifications, DNA methylation, and the expression of microRNAs (miRNAs) [4]. MiRNAs, characterized as non-coding RNAs with 18-25 nucleotides, play pivotal roles in modulating cellular behaviors and influencing pathological processes through post-transcriptional regulation [5,6]. Remarkably, over 30% of human mRNAs can be subject to regulation by miRNAs, highlighting their pervasive impact on gene expression [7,8].

While previous research has explored the involvement of miR-18 in various cellular processes, its role in the context of NAFLD has garnered attention. Notably, studies have demonstrated that miR-18 enhances radiotherapy sensitivity in cervical cancer cells by downregulating ATM [9]. In colorectal cancer cells, miR-18 has been reported to impede DNA repair mechanisms by inhibiting ATM, shedding light on its multifaceted functions [10]. Furthermore, investigations into liver cancer have identified the regulatory role of miR-18 [11]. However, in this study, the primary focus is directed towards elucidating the specific contribution of miR-18 to the intricate landscape of NAFLD development.

In summary, the escalating prevalence of NAFLD, coupled with its complex pathogenesis, necessitates a deeper understanding of the molecular players involved. MiRNAs, particularly miR-18, emerge as key regulators, and this study aims to unravel their specific role in the intricate network of events leading to NAFLD. As we delve into the unique contribution of miR-18, we anticipate shedding...
light on potential therapeutic avenues and strategies for effectively managing this prevalent metabolic liver disorder.

2. Mateiass and methods

2.1. Liver sample collection

Liver samples were collected from NAFLD patients and controls in The Quzhou Affiliated Hospital of Wenzhou Medical University. Samples were pathologically confirmed and stored in liquid nitrogen. This study got approval from the Ethics Committee of The Quzhou Affiliated Hospital of Wenzhou Medical University and was conducted after informed consent of each subject.

2.2. Experimental mice

A total of 36 C57BL/6JC mice at 7 weeks old (Charles River Laboratories, Beijing, China) were habituated for one week and then 9 mice were randomly selected into normal diet group (ND) and the remaining were in high-fat diet group (HFD). Mice in ND group were fed a diet containing 70% carbohydrate, 10% fat and 20% protein, with a total calories of 348 kcal / 100 g. HFD contained 20% carbohydrate, 60% fat and 20% protein, with a total calories of 524 kcal/100g. Mouse feed was provided by SYSE, Co., Ltd, (Changzhou, China). Food ration and fasting body weight of each mouse were daily and weekly recorded, respectively. Liver tissues were collected in 9 mice of ND group and 9 of HFD group in the 4th, 8th and 12th week after sacrifice.

In addition, 18 mice feeding HFD were randomly assigned into two groups, and they were administrated through the tail vein with miR-18 inhibitor or miR-18 NC for 6 weeks. All mice were sacrificed to harvest liver tissues. This study was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

2.3. Blood sample collection

Mice were fast overnight and peritoneally anesthetized with 1% pentobarbital sodium (60 mg/kg). Blood was collected from the angular vein and centrifuged at 3000 rpm, 4°C for 20 min. The upper layer serum was collected for analyzing serum levels of AST, ALT, TG and TC using the automatic analyzer.

2.4. Hematoxylin and eosin (H&E) staining

Liver tissues were fixed in 4% paraformaldehyde for 24 h, which was then dehydrated by gradient ethanol, 24 h, and embedded in paraffin. Tissue slice was used for H&E staining (Boster, Wuhan, China). Food ration and fasting body weight of each mouse were daily and weekly recorded, respectively. Liver tissues were collected in 9 mice of ND group and 9 of HFD group in the 4th, 8th and 12th week after sacrifice.

In addition, 18 mice feeding HFD were randomly assigned into two groups, and they were administrated through the tail vein with miR-18 inhibitor or miR-18 NC for 6 weeks. All mice were sacrificed to harvest liver tissues. This study was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

2.5. Insulin tolerance test (ITT)

Mice were fast in the morning for 4 hours and they had free access to water. In the afternoon, each mouse was weighed and administrated with diluted insulin. Blood glucose was tested at 0, 30, 60, 90 and 120 min, respectively.

2.6. Glucose tolerance test (GTT)

Mice were fast one day prior to GTT and 16 hours later, they were given to free access to water. Each mouse was peritoneally administrated with 0.01 mL/g 20% glucose, with a 1 min interval between each. Blood glucose was tested before administration and 30, 60, 90 and 120 min after glucose administration, respectively.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Liver tissues (30 mg) or cells were lysed in 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA). After centrifugation at 12000 g for 5 min, the supernatant was incubated with 200 μL of chloroform. Fifteen minutes later, the mixture was centrifuged again, and the supernatant was incubated with the same volume of isopropanol. The precipitant after centrifugation was washed in 1 mL of 75% ethanol and centrifuged. At last, the precipitant was air-dried, dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) and stored at -80°C. 0.05 μg RNA was reversely transcribed to complementary deoxyribose nucleic acid (cDNA) and subjected to qRT-PCR. FAS: F: 5′-CTGTTCTTACGTCGGTGC-3′; R: 5′-CTG-TGCAGTTCCCTAGCTTTCC-3′; HMGCR: F: 5′-TGAT-TGACCTTTCCAGGACAG-3′, R: 5′-CTAAAATTGCCATTCCACGAGC-3′; IGF1: F: 5′-GCTCTT-CAGTTCGTTGTTGGA-3′, R: 5′-GCCCTCCTTAGATCACAAGCTCC-3′.

2.8. Cell transfection and treatment

LO2 cells were transfected with miR-18 inhibitor or NC (GenePharma, Shanghai, China) using Lipofectamine 2000. Fresh medium was replaced at 6 h. Transfected cells for 24 h were incubated in FFA mixture (oleate and palmitate at the ratio of 2:1) for another 24 h.

2.9. Luciferase assay

Based on the 3’UTR of IGF1, wild-type and mutant-type IGF1 vectors were constructed. Cells were co-transfected with 50 pmol/L miR-18 mimics/NC and 80 ng IGF1 vectors for 48 h, respectively. Cells were lysed and subjected to measurement of luciferase activity (Promega, Madison, WI, USA).

2.10. Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on PVDF membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

2.11. Statistical analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. All data were expressed as mean ± SD (standard deviation). The paired two-tailed t-test was used to compare differences between two groups. p<0.05 was considered to be statistically significant.

3. Results

3.1. MiR-18 was upregulated in NAFLD samples

Compared to normal liver tissues, miR-18 was upregulated in liver tissues collected from NAFLD patients (Figure 1A). Similarly, highly expressed miR-18 was observed in mice of HFD group than in controls at 4, 8 and 12 weeks, respectively (Figure 1B). With the prolongation of HFD feeding, miR-18 was gradually upregulated. In mice feeding HFD and administrated with miR-18 inhibitor, 238
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3.2. Knockdown of miR-18 alleviates hepatic steatosis and IR

Serum levels of AST, ALT, TG and TC were markedly reduced after in vivo knockdown of miR-18 in mice feeding HFD (Figure 2A-2D). H&E staining revealed pronounced hepatic steatosis in mice of HFD group than controls, and this pathological lesion was markedly alleviated by knockdown of miR-18 (Figure 2E). Impaired glucose tolerance was observed in HFD group (Figure 2F). In addition, IR was seen in HFD group, and it was improved by knockdown of miR-18 (Figure 2G).

3.3. In vitro knockdown of miR-18 regulated lipid metabolism and insulin signals

FFA mixture induction markedly upregulated miR-18 in LO2 cells, which was downregulated by transfection of miR-18 inhibitor (Figure 3A). Relative levels of AST, ALT, TG and TC were higher in culture medium of cells incubated with FFA mixture (Figure 3B-3E). The above-enhanced trends were abolished by knockdown of miR-18. In addition, mRNA levels of FAS and HMGCR, the two vital genes that are associated with the insulin signal, were upregulated following FAS mixture induction, and then abolished by silencing miR-18 (Figure 3F).

3.4. MiR-18 directly bound IGF1

Binding sequences in the 3’UTR of miR-18 and IGF1 were predicted (Figure 4A). It is shown that IGF1 was lowly expressed in liver tissues of NAFLD patients, which was contrary to that of miR-18 (Figure 4B). A negative correlation was identified between expression levels of miR-18 and IGF1 in NAFLD patients (Figure 4C). In addition, IGF1 level was negatively regulated by miR-18 in LO2 cells (Figure 4D, 4E). Based on the predicted binding sequences, we constructed wild-type and mutant-type IGF1 vectors. MiR-18 was capable of negatively regulating luciferase activity in wild-type IGF1 vector, verifying the binding between miR-18 and IGF1 (Figure 4F, 4G).

4. Discussion

The prevalence of NAFLD increases each year, which has become a public health problem [12]. NAFLD is a...
complex multifactorial disease, involving different genetic, environmental, and metabolic factors. It is closely related to obesity, diabetes, and metabolic syndromes [13,14]. The liver exerts a fundamental role in lipid metabolism. Fatty liver is the result of lipid accumulation in liver cells. Recent epigenetic researches have shown that miRNAs are linked to obesity, IR, and NAFLD [15].

Through degrading or inhibiting translation of mRNAs, miRNAs negatively regulate expressions and functions of target genes [16-18]. Accumulating evidences have illustrated the involvement of miRNAs in the development of NAFLD. MiR-122 is abundantly expressed in the liver, which is a vital regulator in lipid metabolism [19,20]. It is reported that miR-185 contributes to maintaining fatty acid metabolism and cholesterol homeostasis, as well as improves IR[21]. Knockdown of miR-21 alleviates impaired glucose tolerance, steatosis and obesity in mice feeding HFD [22]. MiR-34a is one of the miRNAs that are the most sensitive to lipid response. MiR-34a is upregulated in mice feeding HFD and positively correlated to the severity of human fatty liver [23]. Our findings uncovered that miR-18 was upregulated in both NAFLD patients and mice feeding HFD.

Owing to the specific regulation and stable expression, miRNAs may be utilized as therapeutic targets in diseases [24]. Knockdown of miR-21 inhibits metastasis and induces apoptosis and necrosis in hepatocellular cancer (HCC) cells [25]. The role of miR-21 in regulating HCC growth is also identified. As a result, miR-21 is a promising target in the treatment of HCC. In this paper, in vivo knockdown of miR-18 in mice feeding HFD markedly alleviated liver steatosis and IR. We believe that miR-18 can be used as a hallmark of NAFLD.

5. Conclusions

MiR-18 is upregulated in NAFLD patients and mice. Knockdown of miR-18 alleviates HFD-induced hepatic steatosis and IR through interacting with IGF1 to regulate lipid metabolism and insulin signals.

Conflict of interest
The authors declared no conflict of interest.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
This study was approved by the ethics committee of The Quzhou Affiliated Hospital of Wenzhou Medical University. This study was approved by the Animal Ethics Committee of Wenzhou Medical University Animal Center.

Informed consent
Signed written informed consents were obtained from the patients and/or guardians.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions
HY and CY designed the study and performed the experiments, HY and BZ collected the data, CY and BZ analyzed the data, HY and CY prepared the manuscript. All authors read and approved the final manuscript.

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