Up-regulation of microRNA-367 promotes liver steatosis through repressing TBL1 in obese mice

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Abstract. – OBJECTIVE: Increasing evidence has demonstrated that microRNAs (miRNAs) play a critical role in the progression of metabolic disorders, including obesity-induced non-alcoholic fatty liver disease (NAFLD). In the present study, the expression and function of miR-367 were investigated.

MATERIALS AND METHODS: C57BL/6 male mice aged 8 weeks were fed with a normal diet (ND) or high-fat-diet (HFD). The expression levels of miR-367 were analyzed in livers from two groups of mice by quantitative real-time PCR. Adenovirus containing miR-367 or negative control (NC) were constructed and administrated into C57BL/6 mice by tail vain injection. Potential targets of miR-367 were screened by miRWalk software and luciferase reporter assays. Mutagenesis analysis and Western blots were used to further determine the target of miR-367 in obese mice.

RESULTS: We found that the expression of hepatic miR-367 was up-regulated in obese mice. In vitro and in vivo studies further demonstrated that overexpression of miR-367 mimics promoted triglyceride accumulation in cells and lean mice. At the molecular level, transducin beta-like 1 (TBL1), a coactivator of nuclear receptor peroxisome proliferator-activated receptor (PPAR) α, was identified as a direct target of miR-367. As a result, miR-367 overexpression resulted in an inhibition of fatty acid oxidation, leading to hepatosteatosis.

CONCLUSIONS: Our data suggest miR-367/TBL1 regulatory pathway might have an important role for in the development of NAFLD.

Key Words: Obesity, Liver steatosis, MicroRNA, miR-367, TBL1, PPARα.

Introduction

Non-alcoholic fatty liver disease (NAFLD), featured by an aberrant accumulation of triglycerides (TGs) in hepatocytes, is tightly associated with the development of metabolic syndrome and liver diseases, including type 2 diabetes, coronary heart disease, liver fibrosis and hepatocellular carcinoma (HCC). Although it has been well-established that obesity is an important risk factor for NAFLD, the molecular mechanisms are still incompletely understood.

MicroRNAs (miRNAs) belong to a class of small and non-coding RNAs and inhibit gene expression by binding to the 3′-untranslated region (UTR) of target mRNA, leading to transcriptional repression or degradation of target mRNA. Recent studies have shown that dysregulation of several miRNAs is associated with the development of obesity-induced NAFLD, while their biological roles in the pathogenesis remain to be explored.

The expression and roles of miR-367 have been well-established in several types of human cancers. For instance, Campayo et al. reported that high expression levels of miR-367 are associated with unfavorable prognosis in resected non-small cell lung cancer. Besides, miR-367 negatively regulates apoptosis induced by adriamycin in osteosarcoma cells by targeting KL. F4. However, until now, the function of miR-367 in other biological events, inducing hepatic TG metabolism, has not been investigated.

Materials and Methods

Animal Studies

Male C57BL/6 mice, ob/ob mice and db/db mice aged 8 weeks were purchased from the Shanghai Laboratory Animal Company (SLAC, Shanghai, China). For high-fat-diet studies, C57BL/6 were fed with 60% kcal fat-containing diet (Research Diets, New Brunswick, NJ, USA) or 10% kcal fat (Research Diet) as a control. All animal experimentations were approved by the Animal Care Committee of Henan University.
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**Cell Culture, Transfection and Luciferase Assays**

HepG2 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China). Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and maintained at 37°C in a humidified atmosphere with 5% CO₂. All transfections were performed by using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For luciferase assays, cells were seeded in 24-well plates and transfection efficiency was normalized by co-transfecting Simian virus 40 (SV40) plasmids (Promega, Madison, WI, USA). Luciferase values were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

**Cell Culture, Transfection and Luciferase Assays**

**Real-time PCR Analysis**

Total RNA from tissues and cells was extracted using the TRIzol Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Premix Ex Taq reagents (TaKaRa, Dalian, China). Relative expression levels of miR-367 were calculated using the 2^{-ΔΔCt} method with U6 as the endogenous reference gene.

**Western Blots**

Cells and tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM β-Mercaptoethanol, 2% w/v SDS, 10% glycerol). After centrifugation at 10,000×g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were performed using primary antibodies targeting TBL1, PPARα and GAPDH (Abcam, Cambridge, MA, USA). GAPDH was used as a loading control. The proteins were visualized by an ECL chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

**Statistical Analysis**

Data were expressed as mean±standard error of the mean (SE). The analysis was conducted with GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). Significance between two groups was analyzed using the unpaired two-tailed t-test (*p<0.05, **p<0.01, ***p<0.001)

**Results**

**Increased Expression of miR-367 in Obese Livers**

To explore the relationship between hepatic miR-367 expression and obesity-induced hepatic steatosis, C57BL/6 mice aged 8 weeks were fed with a normal diet (ND) or high-fat-diet (HFD) for 2 or 4 months. As a result, quantitative real-time PCR experiments showed that expression levels of miR-367 were gradually increased in HFD mice (Figure 1A). To further confirm this result, leptin-deficient mice (ob/ob) and leptin receptor-deficient mice (db/db) were used. In agreement, miR-367 was also up-regulated in these two obese mice, compared with age-matched lean mice (Figures 1B-C).

**Overexpression of miR-367 Induced TG Accumulation in Hepatocytes and Livers**

Because of the up-regulation of miR-367 in obese mice, we speculate that it might play a role in the regulation of hepatic TG metabolism. To test this hypothesis, its mimics or negative controls (NC) were transfected into HepG2 cells (Figure 2A). As a result, transfection of miR-367 mimics resulted

Figure 1. Up-regulation of miR-367 in obese livers. (A-C) Relative expression of miR-367 in livers of mice fed a HFD (A), ob/ob mice (B) or db/db mice (C).
in an increased TG contents in HepG2 cells (Figure 2B). Similar results were also observed in mouse primary hepatocytes (MPHs) (Figures 2C-D).

To further explore the roles of miR-367 in vivo, adenoviruses containing miR-367 or NC were constructed and administrated into C57BL/6 male mice by tail vain injection (Figure 3A). The up-regulation of miR-367 was only detected in livers (Figure 3A), but not in other organs, including white adipose tissues, heart, kidney and skeletal muscles (data not shown). 10 days later, mice were sacrificed for analysis. As shown in the Figure 3B, hepatic TG contents were increased, which was further demonstrated by Oil Red O staining (Figure 3C). Besides, serum TG levels were also elevated (Figure 3D), while body weight and food intake remained unaffected (Figures 3E-F). Therefore, our results suggest that overexpression of miR-367 could promote TG accumulation in healthy mice.

**miR-367 Overexpression alters PPARα Signaling**

It has been well-established that hepatic TG homeostasis is tightly controlled by multiple pathways, including de novo lipogenesis and fatty acid oxidation. Therefore, we performed genes expression analysis by quantitative real-time PCR using livers overexpressing miR-367 or NC. As shown in the Figures 4A-B, overexpression of miR-367 led to a markedly down-regulation of genes related to mitochondrial and peroxisomal fatty acid oxidation (Figure 4A), while genes responsible for de novo lipogenesis remained unaffected (Figure 4B). Consistently, reduced serum β-hydroxybutyrate levels were observed in C57BL/6 mice overexpressing miR-367, indicating a deficiency in fatty acid oxidation (Figure 4C). Given that PPARα is a master regulator of fatty acid oxidation, its mRNA and protein expression were examined. Interestingly, expression levels of PPARα were also unchanged by miR-367 overexpression (Figures 4D-E), suggesting that miR-367 might alter PPARα signaling by reducing its transcriptional activity without altering its expression.

**TBL1 is a Direct Target Gene of miR-367**

Therefore, potential targets of miR-367 were further screened by a bioinformatics approach (miRWalk software, Heidelberg, Germany). Here, transcriptional cofactor transducin β-like
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(TBL) 1 was chosen for further experiments since it harbored a miR-367 binding site in its 3’-untranslated region (3’-UTR) (Figure 5A). Moreover, it has been demonstrated that TBL1 acts as a co-activator of PPARα, by which it can regulate hepatic TG homeostasis. To test this hypothesis, 3’-UTR of TBL1 gene was cloned into a luciferase reporter vector and transfected into HepG2 cells. As expected, overexpression of miR-367 mimics led to a reduction in luciferase activity when the reporter construct contained the wild-type 3’-UTR (Figure 5B). However, point mutation of TBL1 3’-UTR largely blocked the inhibitory effect of miR-367 mimics (Figure 5B), suggesting that miR-367 could bind to this seed region to regulate TBL1 expression. In agreement, endogenous protein levels of TBL1 were down-regulated by miR-

Figure 3. Overexpression of miR-367 promotes hepatosteatosis in C57BL/6 mice. A, Relative expression of miR-367 in livers of C57BL/6 mice transfected with adenovirus containing miR-367 or negative control (NC). B, Liver TG contents in two groups of mice after adenovirus delivery for 10 days. C, Representative oil red O staining of liver sections from two groups of mice. D-F, Serum TG levels. D, Body weight. E, Food intake (F) in two groups of mice.

Figure 4. miR-367 regulates PPARα down-stream target genes. (A-B) Relative mRNA levels of genes related to fatty acid oxidation (A) and de novo lipogenesis (B). (C-D) Relative mRNA and representative protein levels of PPARα in mice transfected with adenovirus containing miR-367 or negative control (NC).
367 mimics in HepG2 cells, MPH or livers of C57BL/6 mice (Figures 5C-E), further support a notion that TBL1 is a direct target gene of miR-367.

**Discussion**

In the current study, we revealed for the first time that up-regulation of miR-367 in obese livers could promote TG accumulation and hepatosteatosis. This is supported by multiple lines of evidence. Firstly, miR-367 expression was increased in three models of obese mice, compared with lean littermates. Secondly, ectopic overexpression of miR-367 mimics promoted TG accumulation in hepatocytes and livers. Thirdly, luciferase reporter assays and Western blots were used to show that miR-367 has a direct contact with the 3’-UTR of TBL1, by which miR-367 affects PPARα signaling and fatty acid oxidation. Notably, a recent work reported that miR-367 promotes hepatocellular carcinoma (HCC) cell proliferation by negatively regulates its target gene PTEN. Besides, the expression of miR-367 and PTEN are significantly inverses correlated in HCC patients. Given that fatty liver has been linked to the development of HCC, we speculate that up-regulation of miR-367 in obese livers might be a causal factor to promote HCC initiation and/or progression.

It has been shown that impaired hepatic expression of TBL1 represents a common feature of mono-and multigenic fatty liver mouse models. As a result, ablation of TBL1 gene expression in healthy mice resulted in hypertriglyceridemia and hepatic steatosis, due to reduced fatty acid oxidation. On the other hand, overexpression of TBL1 could improve liver steatosis by co-activation of PPARα to up-regulate its down-stream target genes. Of note, many studies have demonstrated that deficiency in PPARα or its coactivators, such as PGC-1α, Sirt1 and BAF60a, could lead to hepatic TG retention and hyperlipidemia in obese animals and humans. Therefore, a better understanding of the molecular mechanisms that control PPARα expression or transcriptional activity might provide new therapeutic targets for NAFLD and related metabolic disorders.

**Conclusions**

We observed a novel miRNA that regulates hepatic TG homeostasis by modulating PPARα signaling and fatty acid oxidation. However, it remains unknown the molecular determinants for the up-regulation of miR-367 in obese livers. Further investigations are still needed to clarify this question.
Conflict of Interest
The authors declare no conflicts of interest.

References


