Original Article

MicroRNA-802 regulates hepatic insulin sensitivity and glucose metabolism

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Abstract: The expression level of microRNA-802 (miR-802) is increased in livers of high-fat diet (HFD)-fed mice and obese human subjects; however, the function of miR-802 in the development of obesity-associated insulin resistance remains incompletely understood. Here we studied the potential role of miR-802 in regulating hepatic glucose metabolism and insulin sensitivity. Mice were fed either a standard chow diet or HFD for 12 weeks, and then the HFD mice were infected by injection with an adeno-associated virus expressing miR-802 or miR-802-SP. Six weeks after the injection, we measured blood glucose, plasma insulin, and insulin sensitivity in the mice. In addition, hepatic glucose levels and PI3K-Akt pathway gene expression were analyzed. Adeno-associated viral-mediated overexpression of miR-802 in the livers of HFD mice caused impaired glucose homeostasis and insulin sensitivity, thus giving rise to decreased protein level of pAkts473 and pPI3K, and increased protein levels of pPTEN, G6PC, and GluT2. In contrast, loss of miR-802 function in the liver of HFD mice led to increased pAkts473 and pPI3K, and decreased levels of pPTEN, G6PC, and GluT2, thereby improving glucose metabolism and insulin resistance. Our findings confirmed MiR-802 as a regulator of liver glucose metabolism and insulin signaling.

Keywords: microRNA-802, insulin resistance, hepatic, glucose metabolism, high-fat diet

Introduction

Obesity is associated with type-2 diabetes mellitus (T2DM) and the metabolic syndrome, which is characterized by insulin resistance and impaired glucose and lipid metabolism [1, 2]. Insulin resistance represents a hallmark in the development of obesity [3, 4]. The liver plays a critical role in energy metabolism and is a major insulin target organ for glucose homeostasis. In the liver, insulin functions as an important regulator of carbohydrate and lipid homeostasis via a complex signaling network [4, 5].

MicroRNAs (miRNAs) represent a new class of regulatory RNAs that are critically involved in regulating the expression of various genes. Since a single miRNA has multiple target genes, miRNAs could theoretically regulate up to 30% of human protein coding genes [6, 7]. Recent studies indicated that miRNAs are important players in insulin production and secretion, pancreatic islet development, insulin resistance, and diverse aspects of both glucose homeostasis and lipid metabolism mechanisms implicated in T2DM [8]. They also contribute to the pathogenesis of obesity and its related medical complications [9-11]. In a population study that characterized differential miRNA expressions in the liver and plasma of human patients, miR-802 was upregulated in overweight compared with lean individuals. Previous studies also revealed a significant increase in the circulating levels of miR-101, miR-375, and miR-802 in patients with T2DM compared with normal glucose tolerance (NGT). Finally, miR-802 was also upregulated in the liver and brown adipose tissue (BAT) of male C57BL/6J mice fed a high fat diet (HFD) [12].

The present study therefore investigated the close association of miR-802 to obesity and diabetes. The specific mechanism by which this
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Table 1. Primer sequences used in PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACCCACAGACTGGAGATGG</td>
<td>GGAGACACCTGGCTCTCAG</td>
<td>20</td>
</tr>
<tr>
<td>PTEN</td>
<td>AGGCCGACACTGGGTAGTA</td>
<td>GGGAATGGTACTCCCTTGTCT</td>
<td>21</td>
</tr>
<tr>
<td>PI3K</td>
<td>CGAAACAAGGCGAGAACTC</td>
<td>ACCACTAAGGACAGGACATA</td>
<td>20</td>
</tr>
<tr>
<td>AKT</td>
<td>TATCCAGATGGTCGCCAA</td>
<td>GGAGCCACACTTGAATCC</td>
<td>20</td>
</tr>
<tr>
<td>G6PC</td>
<td>CGAGGAAAAGAAGGACC</td>
<td>GTGGGGAAAGTGAGCAG</td>
<td>17</td>
</tr>
<tr>
<td>GluT2</td>
<td>GGACTTGTGCTGGA</td>
<td>GCCCAATCTCAAGAAACTGAC</td>
<td>22</td>
</tr>
</tbody>
</table>

association is mediated remains unclear, and we speculate that miR-802 could regulate glucose metabolism in the liver via key enzymes that affect insulin-signaling pathways and glucose metabolism. In our study, we established an HFD-induced insulin resistance model in mice, and used an adeno-associated viral vector to up- and down-regulate miR-802 expression, in order to investigate the mechanism by which miR-802 modulates insulin resistance and glucose metabolism. In addition, liver tissues were stained with H&E staining to study effects on fat degeneration.

Materials and methods

Mouse models

Male C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mouse lines were originally obtained from The Jackson Laboratory. The mice were housed under specific pathogen-free conditions in a temperature-(20-24°C) and humidity-controlled (45-55%) environment under a 12 h/12 h light/dark cycle. All studies were approved by the Institutional Animal Care and Use Committee of the Centre for Gerontology and Geriatrics of Hebei Province in China.

Six-week-old C57BL/6J mice were fed a standard chow diet (Con, D 12450B, 10% kcal from fat) or a HFD (HFD-D12450B, 60% kcal from fat) from Research Diet for 12 weeks. HFD-fed mice were injected intravenously through the tail vein with an adeno-associated viral vector encoding green fluorescent protein and miR-802 (AAV-miR-802), miR-802 sponges (AAV-miR-802-SP), or a negative control at a dose of 1*10^11 plaque-forming units in 0.1 ml PBS. Six weeks after the vector injections, the mice were anesthetized, and blood was collected via the eyes. The livers were harvested, immediately frozen in liquid nitrogen, and then stored at -80°C until used for analysis.

Adeno-associated viral vector construction

The recombinant adeno-associated virus (AAV) vectors containing GFP expressed four different target genes: miR-802 (upregulates the expression of miR-802), AAV-miR-802-SP: complementary sequences with miR-802 (downregulates the expression of miR-802 by sponge sealing), and the respective negative controls (AAV-EGFP and AAV-NC). All were purchased from Shanghai Hanbio Biotechnology Co., Ltd.

Metabolic measurements

Serum insulin levels were analyzed using the Ultra Sensitive Mouse Insulin ELISA Kit (ALPCO Chem. Inc, United States, #04505). Blood glucose levels were measured by Accuchek Active Meter (ACCU-CHEK Roche, Germany). For the glucose tolerance testing (GTT), mice were fasted for 16 h, and then injected intraperitoneally with glucose (2 g/kg BW). Glucose area under the curve (AUC) during GTT was calculated using the trapezoidal integration method. The quantitative insulin sensitivity check index (QUICKI) was calculated as follows: QUICKI = 1/[log(I_0) + log(G_0)] (G_0-fasting glucose (mg dl-1), I_0-fasting insulin (μU ml-1) [13].

Commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to measure plasma triglycerides, cholesterol, and hepatic concentrations of glycogen according to the manufacturer’s instructions.

RNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was extracted from liver tissue using TRIzol reagent (Invitrogen). The quality and the concentration of the RNA were determined by Nanodrop 2000 (Thermo, USA). RNA was reverse transcribed (RT) to cDNA (Promega, USA), and quantitative real-time PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems, USA) using the Syber Green I Go Taqs qPCR Master Mix (Promega, USA).
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MicroRNAs were prepared using miRcute miRNA Isolation kits (Tiangen Biotech Co., Ltd, Beijing, China). Reverse transcription was performed using the miRNA First-Strand cDNA Synthesis System (Tiangen Biotech Co., Ltd, Beijing, China), and microRNA levels were analyzed using miRcute Plus miRNA qPCR Detection Kit (Tiangen Biotech Co., Ltd, Beijing, China).

The relative expression level of miRNA or mRNA was normalized to an internal invariant control, namely U6 small nucleolar RNA or GAPDH. The primer sequences of miR-802 and U6 were purchased from Tiangen Biotech Co., Ltd, as shown in Table 1.

Western blotting

The cytosolic and membrane fractions were extracted from liver tissues, as previously described. Protein was separated by 10% SDS-PAGE, transferred to PVDF membrane (Millipore), blocked with 5% nonfat dry milk, and probed with antibodies at 4°C overnight. The blots were incubated with HRP-conjugated anti-IgG, followed by detection with ECL. Antibodies

Figure 1. The effect of HFD on insulin resistance and increased hepatic expression of miR-802. Mice were fed with HFD or normal control diets for 12 weeks. Measurements were performed during the course of the HFD, as shown below (n = 7). A. Total BW. B. Fasting blood glucose. C. Average daily calorie intake. D. GTT, performed after 11 weeks of HFD. E. Fasting plasma insulin. F. AUC. G. Quicki. H. QRT-PCR analysis of miR-802 expression in the liver of HFD mice and controls. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.005, 2-tailed Student’s t test (B-K).
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against PI3K, phospho-PI3K (Y607), PTEN, phospho-PTEN (Ser380), Akt, phospho-Akt (Ser473), GluT2, and G6PC were all purchased from Cell Signaling (USA) or Abcam (England).

**Histological analysis of tissues**

Pathological changes in the liver were monitored by hematoxylin and eosin (H&E) staining using the H&E kit (Solarbio, Beijing, China). In brief, liver tissue was fixed in 4% paraformaldehyde buffer for 1 h at 37°C. They were subsequently embedded in a liquid paraffin solution on dry ice and cut into 5 μm sections. The slides were first incubated with hematoxylin for 5 min and then washed with 1% ethanol hydrochloride for 3 s. After washing with water, the slides were stained with eosin for 3 min and

Figure 2. Hepatic overexpression of miR-802 aggravated HFD-induced insulin resistance and glucose intolerance in the liver (n = 6). Mice were fed with HFD for 12 weeks, and then infected with an adeno-associated virus expressing miR-802 or AAV-Con. Measurements were performed 6 weeks after the injections, as shown below. A. Expression of AAV in the liver, as shown by fluorescence microscopy (mag, 200×). B. QRT-PCR analysis of miR-802 expression in the liver of AAV-miR-802 mice and AAV-GFP controls. C. Total BW. D. GTT, 6 weeks after injection of AAV. E. AUC. F. Fasting plasma insulin. G. QUICKI. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01 vs. Con mice, #P < 0.05, ##P < 0.01 vs. AAV-GFP mice; 2-tailed Student’s t test.
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Dehydrated with an alcohol gradient. The selected specimens were visualized and photographed under an electron microscope (HITACHI H7500, Japan).

Statistics

The data are represented as the mean ± standard error of mean (SEM). Two-tailed unpaired Student’s t-tests were used for between-group comparisons. The ANOVA multiple comparison test (SPSS 13.0) followed by Tukey’s post hoc test were used for comparisons among groups. P < 0.05 was considered to be statistically significant.

Results

HFD mice exhibited impaired insulin sensitivity and increased hepatic expression of miR-802

After 12 weeks on the HFD, the mouse body weights and fasting blood glucose concentrations were significantly increased compared with those of the control mice (Con group; Figure 1A, 1B). Although no differences were
detected in average daily calorie intake (Figure 1C), HFD-mice showed aggravated glucose intolerance and insulin sensitivity (Figure 1D, 1E). Furthermore, AUC was increased (Figure 1F), whereas QUICKI was decreased (Figure 1G) in HFD mice, indicating that the HFD successfully induced obesity and insulin resistance. Next, we detected increased hepatic miR-802 expression in HFD-fed mice (Figure 1H), demonstrating the critical role of miR-802 in both insulin resistance and glucose metabolism in the liver.

Overexpression of hepatic miR-802 aggravated HFD-induced insulin resistance in mice

To determine if the increased miR-802 expression contributed to the development of insulin resistance, we overexpressed miR-802 with AAV-miR-802 in HFD mice. Six weeks after tail vein injections of AAV-miR-802 or AAV-GFP, we showed by fluorescence microscopy that AAV was successfully expressed in the liver (Figure 2A). Also, the hepatic mRNA expression levels of miR-802 were increased in the AAV-miR-802 group compared with the AAV-GFP group (Figure 2B). As expected, glucose intolerance was developed in the AAV-miR-802 mice (Figure 2D), and both plasma insulin (Figure 2F) and AUC (Figure 2E) were increased, whereas QUICKI (Figure 2G) was decreased, as compared with the AAV-GFP mice, indicating that overexpression of hepatic miR-802 might induce insulin resistance. There was no difference in the body weights of the mice in the two groups (Figure 2C).

Inhibition of miR-802 expression in the liver improved glucose homeostasis and hepatic insulin sensitivity in HFD-fed mice

To address whether miR-802 directly induced insulin resistance, we inhibited miR-802 expression in the liver of HFD-fed mice with AAV-miR-802-SP, a recombinant adenovirus-associated viral expressing an miR-802 sponge. Six weeks after the AAV-miR-802-SP or AAV-NC injection, AAV were successfully expressed in the mice livers (Figure 3A), and the miR-802 levels in the liver of the AAV-miR-802-SP group were reduced (Figure 3B). The levels of plasma insulin (Figure 3F) and AUC (Figure 3E) were also decreased in mice treated with AAV-miR-802-SP, accompanied by improved glucose intolerance (Figure 3D) and increased levels of QUICKI (Figure 3G). The body weights did not differ significantly among the HFD groups (Figure 3C). These changes showed that reduction of hepatic miR-802 expression in HFD mice improved insulin sensitivity and glucose homeostasis.

Expression of hepatic miR-802 interfered with the PI3K-Akt pathway in HFD mice

To investigate the mechanism by which miR-802 affected the insulin sensitivity and glucose metabolism, we measured changes in the PI3K-Akt pathway by quantitative RT-PCR and Western-blot assays, based on miR-802 expression in the liver of HFD-fed mice. Compared with the Con group, the HFD mice showed decreased mRNA expression of PI3K and Akt, and increased mRNA expression of PTEN, G6PC, and GluT2 (Figure 4A). Upregulating the expression of miR-802 induced further decreases in mRNA expression of PI3K and Akt, and further increased mRNA expression of PTEN, G6PC, and GluT2 compared to the AAV-GFP group, while downregulating the expression of miR-802 increased the mRNA expression of PI3K and Akt, and decreased that of PTEN, G6PC, and GluT2 (Figure 4A).

Down- or up-regulation of hepatic miR-802 expression had no effect on total protein level of PTEN, PI3K, and Akt. Compared with the Con group, the protein levels of pPI3K and pAkt were decreased in HFD mice, while the protein levels of pPTEN, G6PC, and GluT2 were increased. Overexpression of hepatic miR-802 led to further decreased protein levels of pPI3K and pAkt, and further increased protein levels of pPTEN, G6PC, and GluT2 compared with the AAV-GFP group. After inhibition of hepatic miR-802 expression, the protein level of pPI3K and pAkt increased, while the protein levels of pPTEN, G6PC, and GluT2 decreased compared with AAV-NC mice (Figure 4B, 4C). We also found that hepatic glycogen was decreased in the AAV-miR-802 mice, but increased in the AAV-SP-miR-802 mice, compared with the respective controls (Figure 4D). The results suggested that miR-802 affected the insulin sensitivity and glucose metabolism via regulation of the PI3K-AKT pathway.

Hepatic expression of miR-802 affect the steatosis in the liver

In this study we also found the expression of miR-802 could impact hepatic steatosis. The
Figure 4. The effect of miR-802 on PI3K-Akt signaling in HFD mice. Mice were fed with HFD for 12 weeks, and then infected with an adeno-associated viral vector encoding green fluorescent protein (AAV-GFP/NC), miR-802, or miR-802 sponges (n = 6). (A, B) The expression levels of phosphoinositide-3-kinase (PI3K), PTEN, AKT, G6PC, and GluT2 were determined by quantitative real-time-polymerase chain reaction (RT-PCR) (A) and Western blotting (B). Phosphorylation levels of PI3K, PTEN, and AKT were assayed by Western blotting (B). (C) Densitometric analysis of protein expression. (D) Concentrations of hepatic glycogen. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.005 vs. Con mice, #P < 0.05, ##P < 0.01, ###P < 0.005 vs. AAV-GFP mice, ΔP < 0.05, ΔΔP < 0.01, ΔΔΔP < 0.005 vs. AAV-NC mice, 2-tailed Student's t test.
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levels of plasma and hepatic TG were elevated in AAV-miR-802 mice, but decreased in AAV-miR-802-SP mice compared with the respective control mice (Figure 5A, 5B). H&E staining showed lipids accumulated in the liver of the mice injected with AAV-miR-802, whereas liver steatosis was alleviated in the mice injected with AAV-miR-802-SP compared with their respective controls (Figure 5D). Meanwhile, no changes were found in the plasma levels of TC across the groups (Figure 5C). These results indicated that hepatic expression of miR-802 might regulate liver steatosis mechanisms.

Discussion

In this study, expression levels of miR-802 were increased in HFD-fed mice livers compared with control mice, suggesting that this microRNA might play a significant role in hepatic glucose metabolism. We also found that upregulating the expression of miR-802 in obese mice contributed to obesity-induced metabolism damage, and that inhibition of endogenous miR-802 in obese mice ameliorated glucose tolerance. Our findings, therefore, imply that modulating miR-802 expression might supply novel opportunities for the treatment of obesity-associated metabolic syndrome.

Hepatic expression of miR-802 is consistently increased in HFD-fed mice and overweight humans. Further, miR-802 is a physical regulator of insulin signaling and plays a crucial role in hepatic glucose metabolism. Overexpression of miR-802 by AAV in mice also impairs glucose homeostasis. In this study, pPTEN expression was increased, but that of pPI3K and pAkt was decreased upon overexpression of miR-802. Conversely, miR-802 silencing caused remarkable increases in the expression of...
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pPI3K and pAkt, but decreases in expression of pPTEN. These results suggested that miR-802 affects the PI3K-Akt pathway, and in turn, liver insulin sensitivity. Further experiments are still needed to verify the direct target gene of miR-802.

In addition, we also found the expression of GluT2 and G6PC were increased in the AAV-miR-802 mice, while decreased upon inhibition of miR-802, and the opposite variation occurred with hepatic glycogen. G6P is a key enzyme for glucose homeostasis, functioning in gluconeogenesis, and G6PC is the catalytic subunit of glucose-6-phosphatase (G6Pase). G6Pase is located in the endoplasmic reticulum (ER) and catalyzes the hydrolysis of G6P to glucose and phosphate in the last step of the gluconeogenic and glycogenolytic pathways [14]. In addition, GluT2 in the liver, kidney and pancreas acts as a bidirectional glucose transporter. Many studies have indicated that over-expression of GLUT2 in hepatic tissue is found in HFD animals [15, 16]. In our study, increased expression of G6PC enhanced hepatic glucose production, which stimulated the expression of GluT2 to transport glucose outside the liver and decrease hepatic glycogen. All these results altered the level of blood glucose, thereby impacting glucose homeostasis.

MiR-802 might reduce the phosphorylation level of Akt indirectly by means of PTEN and PI3K, thereby affecting the insulin signaling pathway and glucose metabolism of the liver. All the results herein demonstrated that miR-802 could regulate insulin signaling and hepatic glucose production; however, further investigation is needed to identify miR-802’s target mRNA and its molecular effector in disease progression.

In this study the level of TG in the liver of overexpressed miR-802 mice was also elevated and HE staining indicated lipidosis. Nevertheless, TG levels were lower in the liver of knockdown miR-802 mice than in the control group, and lipidosis was improved. This suggested that miR-802 might affect liver lipid metabolism, although not the specific mechanism underlying this effect.

Obesity is a major cause of morbidity and mortality, and there is an urgent need for new treatment strategies [17]. MicroRNAs are small RNAs of ~22 nucleotides that regulate gene translation by specifically binding to the 3'-UTR of mRNAs and blocking translation or targeting the transcript for degradation [18, 19]. Many miRNAs are now implicated in glucose metabolism and were considered as therapeutic targets for human diseases, including diabetes [9, 20]. For example, miR-93 could target GLUT4, a glucose transporter found in adipocytes, explaining in part how elevated levels of miR-93 can contribute to insulin resistance [21]. In addition, overexpression of miR-26 improved insulin sensitivity, decreased hepatic glucose production, and reduced fatty acid synthesis in obese mouse models [22]. We now show that miR-802 has impacts on all three hallmarks of obesity, namely insulin resistance, excessive HGP, and elevated lipid synthesis. In addition to the multiple functions of miR-802, it is important to note that overexpression of miR-802 does not alter weight gain, circumventing the major side effect of increasing insulin sensitivity for diabetes therapies. Given the potent role of miR-802 in liver metabolism as well as the above observations, miR-802 represents a promising target for the treatment of obesity-associated metabolic syndrome.

In conclusion, our study indicates that miR-802 plays a key role in hepatic glucose metabolism and insulin signaling. Overexpression of miR-802 in HFD mice contributes to obesity-induced metabolism abnormalities. Conversely, down-regulating miR-802 in obese mice ameliorates obesity-induced metabolism damage. These findings reveal miR-802 as a potential therapeutic target for obesity-associated metabolic syndrome.

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Disclosure of conflict of interest

None.

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