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MiR-149 Compromises the Reactions of Liver Cells to Fatty Acid via its Polymorphism and Increases Non-Alcoholic Fatty Liver Disease (NAFLD) Risk by Targeting Methylene Tetrahydrofolate Reductase (MTHFR)

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Non-alcoholic fatty liver disease (NAFLD) is a worldwide health problem, and microRNA (miRNA) has been reported to be involved in NAFLD. The objective of our study was to explore the effect of polymorphism in miR-149 on the pathogenesis of NAFLD.

Material/Methods: Real-time PCR was performed to explore the effect of long-chain fatty acid (FFA) on the level of miR-149 and methylene tetrahydrofolate reductase (MTHFR). Then in-silicon analysis and luciferase assay were investigated to verify MTHFR was the target gene of miR-149. Finally, Western-blot analysis and real-time PCR were performed to confirm the control of MTHFR by miR-149.

Results: In this study, we found that miR-149 was apparently upregulated in hepatocytes genotyped as TT treated with FFA; and MTHFR in hepatocytes genotyped as TT treated with FFA was evidently downregulated compared to control. Whereas, FFA had no obvious effect on MTHFR level in hepatocytes genotyped as CC. We searched an online miRNA database and found that miR-149 was a regulator of MTHFR expression, which was confirmed by luciferase assay. In hepatocytes genotyped as TT and treated with or without FFA, miR-149 mimic dose-dependently decreased the level of MTHFR, and miR-149 inhibitor dose-dependently increased the level of MTHFR. And in hepatocytes genotyped as CC treated with or without FFA exhibited a similar inhibition effect of miR-149 on expression of MTHFR.

Conclusions: The data suggested that the polymorphism in miR-149 played an important role in the development of NAFLD via altering the expression of miR-149 as well as its target, MTHFR.

MeSH Keywords: Fatty Acids • Fatty Liver • MicroRNAs

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Background

Non-alcoholic fatty liver disease (NAFLD) is a group of liver disorder featured by the presentation of steatosis and ectopic fat in the liver which cannot be ascribed to alcohol consumption. It varies from simple fatty liver to non-alcoholic steatohepatitis (NASH) that can have various magnitudes of fibrosis and advance to end-stage liver disorder and liver cirrhosis. NAFLD has been an epidemic liver disorder since 21st century. It is estimated that NAFLD occurs in approximately one billion people worldwide [1]. Indeed, it is the top contributor to changed aminotransferases in the Western countries, where NAFLD affects one-third of the population. Recent studies suggested similar morbidity of NAFLD in Asia, where the incidence is similar in urban areas in the Indian continent, though much lower (below 10%) in rural areas [2–5].

The hepatic pathogenesis of NAFLD primarily includes simple steatosis, NASH, cirrhosis, and fibrosis [6]. NASH is noted by hepatocellular inflammation and damage [6]. The polymorphisms of a range of genes, including methylene tetrahydrofolate reductase (MTHFR), leptin receptor (LEPR), and patatin-like phospholipase domain-containing 3 (PNPLA3), have been demonstrated to be implicated in the genetic predisposition to NAFLD [7]. For the MTHFR gene, studies reported contradictory findings with regards to its possible association with NAFLD [8]. The diagnosis of NAFLD was established or ruled out by using ultrasonogram. Thereby, we conducted a meta-analysis as an effective tool for analyzing and integrating the contradictory data from various studies, to highlight the polymorphisms of human MTHFR and evaluate its genetic correlation with NAFLD risks [9].

As small non-coding RNA molecules consisting of ~18–25 nucleotides in length when mature, microRNAs (miRNAs) have post-transcriptionally regulatory role in messenger RNA (mRNA) expression. This happens mainly via the binding to the seed area (2–8 nucleotides) of a miRNA, as composition of the RNA-triggered silencing complex, to complementary sequences situated in the 3' UTR of a target mRNA followed by consequent translational suppression and/or degradation of the mRNA transcript. MiRNAs have now been shown to be involved in an extensive array of biological processes such as cell division, and cell growth and development [10].

Recently, a study performed in Japan by Yamada et al. further supported the potential to apply circulating miRNAs as NAFLD biomarkers [11]. In fact, a human study by Cermelli et al. found that serum levels of miR-34a and miR-122 were substantially higher in people with NASH, indicating a positive association with inflammation and fibrosis stage [12]. Recently, a human study of NAFLD patients revealed elevated levels of hepatic and serum miR-122 in slight to severe steatosis [13].

In 2007, bioinformatic analyses predicted that this pathology was related to the onset of SNPs in miRNAs or miRNA target sites (miR-SNPs), which was confirmed by Wu et al. in an experiment including a variety of human cancers [14,15]. The expression of a range of diverse genes might be affected by SNPs in a miRNA-coding sequence, for instance contributing to a maturation process or compromising processing of the miRNA. On the other hand, SNPs in target sites can either form new binding sites or regulate existing binding sites exhibiting their effects on one or a few specific target molecules. MTHFR is a possible target of miR-149, providing the fact that MTHFR has functional association with the pathology of NAFLD, and the fact that the expression of miR-149 was decreased by the presentation of rs2292832 [16]. In this report, we confirmed the MTHFR/miR-149 association in liver cells treated with FFA, a cell model of NAFLD [17], and determined the relationship between the onset of NAFLD and polymorphism of miR-149 rs2292832.

Material and Methods

Collection and culture of primary liver cells

The liver tissue samples with no known NAFLD were collected in our hospital and cut into pieces. Then 4 mg/mL dispase (Sigma-Aldrich, St Louis, MO, USA) and 3 mg/mL collagenase type I (Sigma-Aldrich, St Louis, MO, USA) were used to digest the samples at 37°C for 60 minutes following the supplier's recommendation. A 70-mm strainer (Falcon; Corning Life Sciences, Tewksbury, MA, USA) was used to pass the cells yield single-cell suspensions, and then DMEM/F12 (Gibco, Auckland, NZ) including 10% FBS (fetal bovine serum) (Invitrogen, Carlsbad, CA, USA) was used to maintain the liver cells under a humidified atmosphere of 5% CO_2/95 air at 37°C, and changed the growth medium every two days up to the cells were cloned, when the cells were grown to 80% confluence, passaged the cells, and cells at passage two were used in the present study. The study protocol was approved by the ethical committee at The Affiliated Hospital of Shaanxi University of Chinese Medicine.

Genotyping

The genomic DNA was extracted from the liver tissue and the chromosome segment containing the rs2292832 polymorphism was amplified using PCR. The PCR product was purified and sent for sequencing to determine the genotype.

Long-chain fatty acid treatment

Culture medium supplemented with 1 mM FFA and 1% BSA (bovine serum albumin) (Keygen, Nanjing, Jiangsu Province,

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China) was utilized to incubate the cells overnight. Each test was repeated three times.

To establish an *in vitro* cell model of NAFLD, primary liver cells were exposed to FFA prepared in culture medium containing 1% BSA (Keygen, Nanjing, Jiangsu Province, China) at a final concentration of 1 mM for 24 hours.

RNA isolation and real-time PCR

TRIzol (Invitrogen, CA, USA) was utilized to isolate total RNA from liver cells in accordance with the supplier's recommendation. The DNase-treated total RNAs was subjected to reverse transcribed with oligo primer (Takara, Japan) containing the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) in accordance with manufacturer's recommendation. TaqMan amplification kits (Applied Biosystems, Foster City, CA, USA) was performed to amplify the cDNA in accordance with manufacturer's protocol. PrimeScript® miRNA RT-PCR Kit (Takara, Japan) was used to perform real-time PCR in order to analyze the expression of miR-149 and MTHFR mRNA based on the protocol of the supplier. RNU43 was used as the internal control to normalize the expression of MTHFR mRNA. Delta-delta-Ct method was used to analyze the expression of MTHFR mRNA and miR-149. All experiments were run three times.

Cell culture and transfection

High glucose-DMEM (Hyclone, Logan, UT, USA) containing 10% FBS (fetal bovine serum), 100 mg/mL streptomycin and 100 U/ml penicillin was utilized to maintain the liver cells with a humidified atmosphere of 5% CO_2/95 air at 37°C. Then 2×10^5 cells/well were cultured in 48-well plates for 12 hours in order to perform transfection, lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) was used to transfected the cells with miR-149 mimics or inhibitors (30 or 60 nM), negative control (i.e., scramble control, a sequence with no known target in human genome procured from Gene Pharma, Shanghai, China) in accordance with manufacturer's instruction. Three independent experiments were repeated.

Luciferase assay

We amplified the full-length 3' UTRs of MTHFR, and subcloned them into the multiple restrictive sites of the psiCHECK-2 plasmid (Promega, Madison, WI, USA) and the Renilla luciferase-coding sequence. An EZ change site-directed mutagenesis kit (Enzynomics, Daejeon, South Korea) was employed to introduce mutations into the seed sequences of MTHFR 3'UTR. We seeded liver cells in a plate of 96 wells (5×10^3 cells per well). After 24 hours, the cells were co-transfected with a psiCHECK reporter vector containing MTHFR and miR-149. Then 48 hours after transfection, the cells were assayed for luciferase activity using a Dual-Glo luciferase reporter assay system (Promega, Madison, WI, USA). Firefly luciferase activity was used to normalize Renilla luciferase activity for each sample.

Western blot analysis

For analysis of the expression of MTHFR, RIPA buffer (Invitrogen, CA, USA) was used to extract the whole protein from liver cells and tissue samples in accordance with manufacturer's instruction. SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) was used to separate the cellular proteins extracted, which were then transferred to nitrocellulose membranes (Immobilin-P; Millipore, Bedford, MA, USA). Then 5% nonfat milk was used to block the membranes for two hours at room temperature, and the anti-MTHFR antibody at a dilution of 1: 5,000 (rabbit, IgG, sc-368720, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to incubate the membrane at 4°C overnight, and then the secondary antibody anti-rabbit IgG at a 1: 10,000 dilution (goat anti-rabbit IgG-HRP, sc-2030, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to detect the primary antibody for another one hour. An enhanced chemiluminescence (Amersham-Pharmacia Biotech, Beijing, China) was used to visualize the specific protein band. And α-actin was served as internal control, each experiments was carried out at least three times.

Statistical analysis

All data were shown as mean ±SEM, and independent student test or one-way ANOVA was used to assess the differences between groups. SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. The value of p less than 0.05 was considered significant.

Results

MiR-149 was increased in hepatocytes genotyped as TT treated with FFA

It has been reported that FFA was utilized to establish NAFLD cell models *in vitro*. In the present study, the effect of FFA on the expression of miR-149 in hepatocytes genotyped as TT or CC was determined using real-time PCR. As shown in Figure 1A, the level of miR-149 in hepatocytes genotyped as TT with treatment of FFA were evidently increased in comparison to control ($p<0.01$). Whereas shown in Figure 1B, the level of miR-149 in hepatocytes genotyped as CC treated with FFA were comparable to the control ($p>0.05$). This suggested that FFA upregulated the expression of miR-149 in hepatocytes genotyped as TT.

MTHFR was the virtual target of miR-149

In the present study, we aimed to understand the role and molecular mechanism of miR-149 in the pathogenesis and

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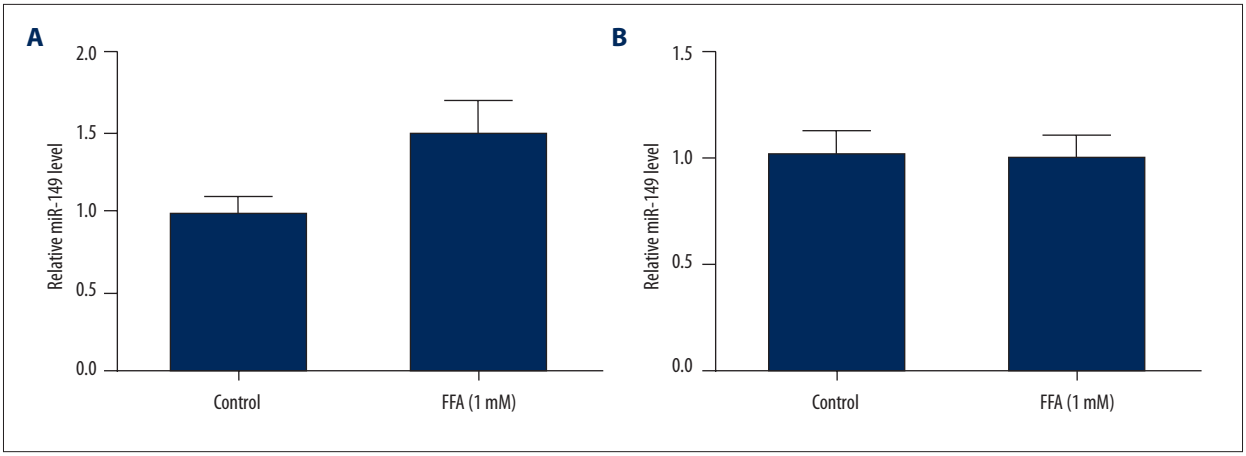

Figure 1. (**A**) miR-149 was up-regulated by FFA in hepatocytes genotyped as TT; (**B**) FFA had no effect on miR-149 level in hepatocytes genotyped as CC.

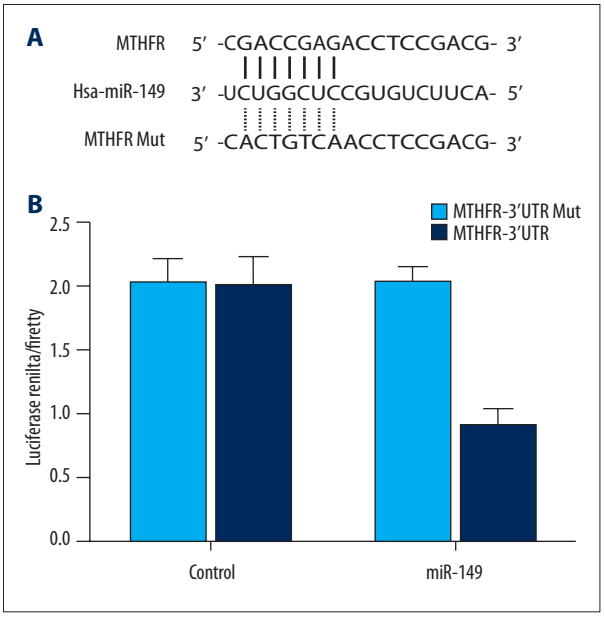

Figure 2. (**A**) MTHFR was identified as target of miR-149, with its possible binding region in MTHFR 3'UTR; (**B**) luciferase assay showed that only the luciferase activity in cells transfected with miR-149 mimics and wild-type 3'UTR of MTHFR were significantly reduced.

progression of NAFLD. We searched using online miRNA target prediction tools to explore the regulatory gene of miR-149 and consequently identified MTHFR as the candidate target gene of miR-149 in cells with the "seed sequence" in the 3'UTR (Figure 2A). The "seed sequence" located on the MTHFR 3'UTR is highly conserved among the species, indicating that this "seed sequence" may play a very important role in the progression of human NAFLD. Furthermore, to verify the regulatory relationship between miR-149 and MTHFR, we applied luciferase activity reporter assay to hepatocytes genotyped as TT. We inserted the fragment of MTHFR 3' UTR with the binding site of miR-149 and targeting sequence mutated into psiCHECK-2 plasmid. The cells were co-transfected with a psiCHECK reporter vector containing wild-type or mutant MTHFR 3' UTR and miR-149 mimic. As shown in Figure 2B, the luciferase activity of wild-type 3'UTR of MTHFR was notably inhibited by miR-149 ($p<0.01$), while the luciferase activity of mutant 3'UTR of MTHFR showed no obvious difference compared to the control ($p>0.05$). This confirmed that miR-149 was the direct regulator of MTHFR and the negative regulator of the expression of MTHFR.

MTHFR was decreased in hepatocytes genotyped as TT treated with FFA

Furthermore, the effect of FFA on the expression of MTHFR in hepatocytes genotyped as TT or CC was determined using Western-blot analysis and real-time PCR, as shown in Figure 3A. There was an evident decrease in mRNA and protein levels of MTHFR of hepatocytes genotyped as TT treated with FFA compared to the control ($p<0.01$). As shown in Figure 3B, the level of MTHFR in hepatocytes genotyped as CC showed no significant difference to the control ($p>0.05$), indicating that FFA regulated MTHFR via an effect on the level of miR-149.

MiR-149 attenuated the expression of MTHFR in hepatocytes genotyped as TT or CC treated with or without FFA

To further validate the hypothesis of the negative regulatory relationship between miR-149 and MTHFR, and whether FFA mediated expression of MTHFR though SNP on miR-137, we investigated the mRNA/protein expression levels of MTHFR of hepatocytes genotyped as TT treated with or without FFA and transfected with different concentration of miR-149 mimics and inhibitors with the use of Western-blot analysis and real-time PCR. As shown in Figure 4A and 4B, cells treated with

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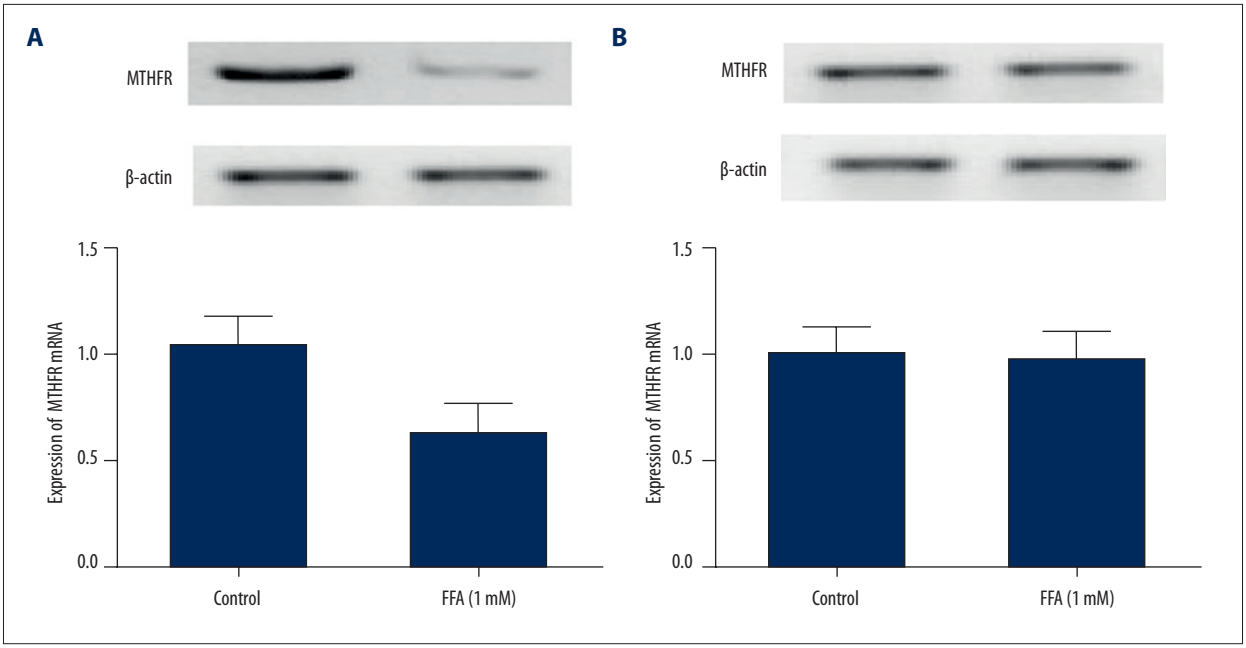

Figure 3. (**A**) MTHFR was downregulated by FFA in hepatocytes genotyped as TT; (**B**) FFA had no effect on MTHFR level in hepatocytes genotyped as CC.

or without FFA displayed a higher mRNA and protein levels of MTHFR subsequent to transfect with 30 nM miR-149 mimic, and displayed an even higher mRNA and protein levels of MTHFR following introduction of 60 nM miR-149 mimic compared to the control and 30 nM miR-149 mimic treatment ($p<0.01$). Whereas in Figure 4C and 4D, the mRNA and protein levels of MTHFR in cells treated with or without FFA was notably higher after transfection with 30 nM miR-149 inhibitor compared to the control, and even higher after transfection with 60 nM miR-149 inhibitor compared to the control and 30 nM miR-149 inhibitor treatment ($p>0.05$). This suggested that miR-149 dose-dependently attenuated the expression of MTHFR in hepatocytes genotyped as TT treated with or without FFA.

In hepatocytes genotyped as CC treated with or without FFA, the trend of miR-149 caused an inhibition of expression of MTHFR was similar with that in hepatocytes genotyped as TT treated with or without FFA ($p<0.01$), and miR-149 mimic decreased the level of MTHFR, and miR-149 inhibitor increased the level of MTHFR in a dose-concentration fashion ($p<0.01$). These data collectively indicated that miR-149 reduced the expression of MTHFR, and FFA mediated expression of MTHFR though SNP on miR-149 (Figure 5).

Discussion

MiRNAs, as a potential regulator of up to 30% of human genes, and have been repeatedly reported to be involved in various human diseases [11–13]. One recent study suggested implication of miRNA in the pathogenesis of NAFLD [18]. MiRNA microassay data showed that miR-149 was substantially elevated in mouse livers with NAFLD, revealing that increased expression of miR-149 might be a factor for NAFLD [19]. MiR-149 has been identified as a tumor inhibitor which could suppress cell-cycle progression and cell proliferation in certain type of cancers [20]. In a recent study, it was shown that miR-149 increased in the hepatic tissues of mice with NAFLD triggered by HFD [19]. Intriguingly, an earlier study showed that downregulation of miR-149 was observed in skeletal muscles in HFD-fed mice [21]. Thereby, FGF-21 has been increasingly considered as a potential interventive tool for the treatment of metabolic disorders such as NAFLD [22]. As miR-149 has been validated as a regulator of FGF-21, it has been demonstrated that the suppressive role of miR-149 in lipogenesis via targeting FGF-21. Additionally, some other miRNAs, such as miR-212, also target FGF-21 in NAFLD [18]. MiR-149 mainly acts as a tumor-suppressor, and its expression is reduced in some cancers such as non-small-cell lung cancer, head and neck squamous cell carcinoma, gastric cancer, colorectal cancer and breast cancer [20–25]. In addition, SIRT1 has been found to a potential therapeutic target in the treatment of HAFLD [26] In this study, we found that MTHFR may function as the candidate target gene of miR-149 based on the result of in-silicon analysis, and we found that wild-type 3'UTR of MTHFR luciferase activity was notably attenuated by miR-149, while mutant 3'UTR of MTHFR luciferase activity remained similar as the control.

Situated at position 36 on the short arm of chromosome 1, the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene

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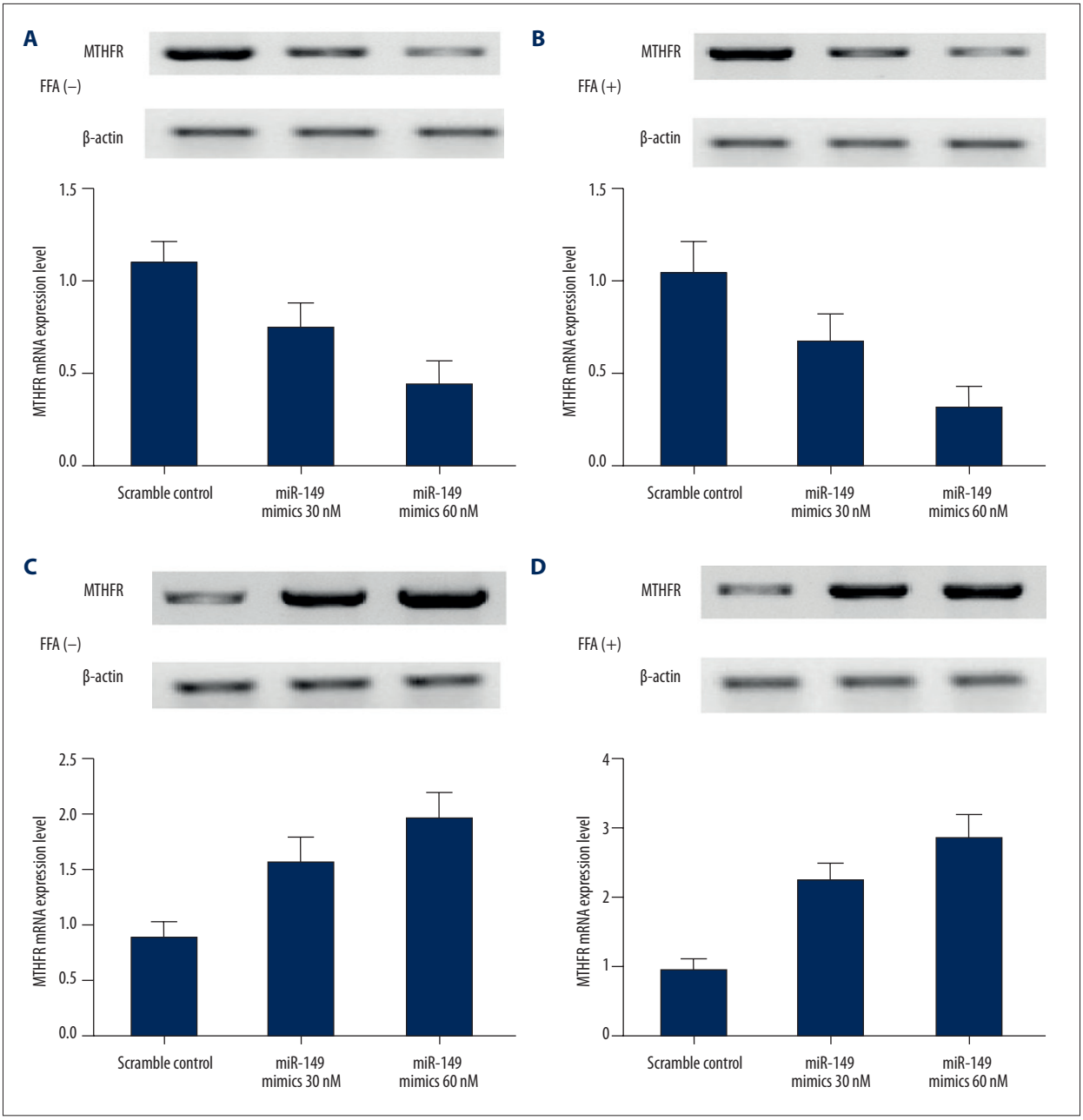

Figure 4. miR-149 attenuated the expression of MTHFR in hepatocytes genotyped as TT treated with or without FFA. (**A**) MTHFR level of cells treated without FFA transfected with 30 and 60 nM miR-149 mimics; (**B**) MTHFR level of cells treated with FFA transfected with 30 and 60 nM miR-149 mimics; (**C**) MTHFR level of cells treated without FFA transfected with 30 and 60 nM miR-149 inhibitors; (**D**) MTHFR level of cells treated with FFA transfected with 30 and 60 nM miR-149 inhibitors.

plays an important role in intracellular folate hemostasis. The enzyme MTHFR is coded by this gene, which acts as catalyzer to irreversibly converse 5,10-MTHFR to a substrate known as 5-metyltetrahydrofolate for methylation from homocysteine to methionine [27]. Many studies have suggested the possible correlation between the most frequent allelic variations of MTHFR gene (A1298C and C677T) and predisposition to a wide range of clinical disorders, including renal failure, heart failure, acute lymphoblastic leukemia, neural tube defects, NAFLD, hepatocellular carcinoma, and gastric cancer [28]. Nevertheless, the role of polymorphisms of MTHFR in the presentation of NAFLD has not been conclusive in studies of various populations [8]. For example, the association between MTHFR A1298C polymorphism and the magnitude of NAFLD was found to be positive in an Italy population [8]. In a Turkish population, A1298C and C677T polymorphisms of MTHFR gene were found to be

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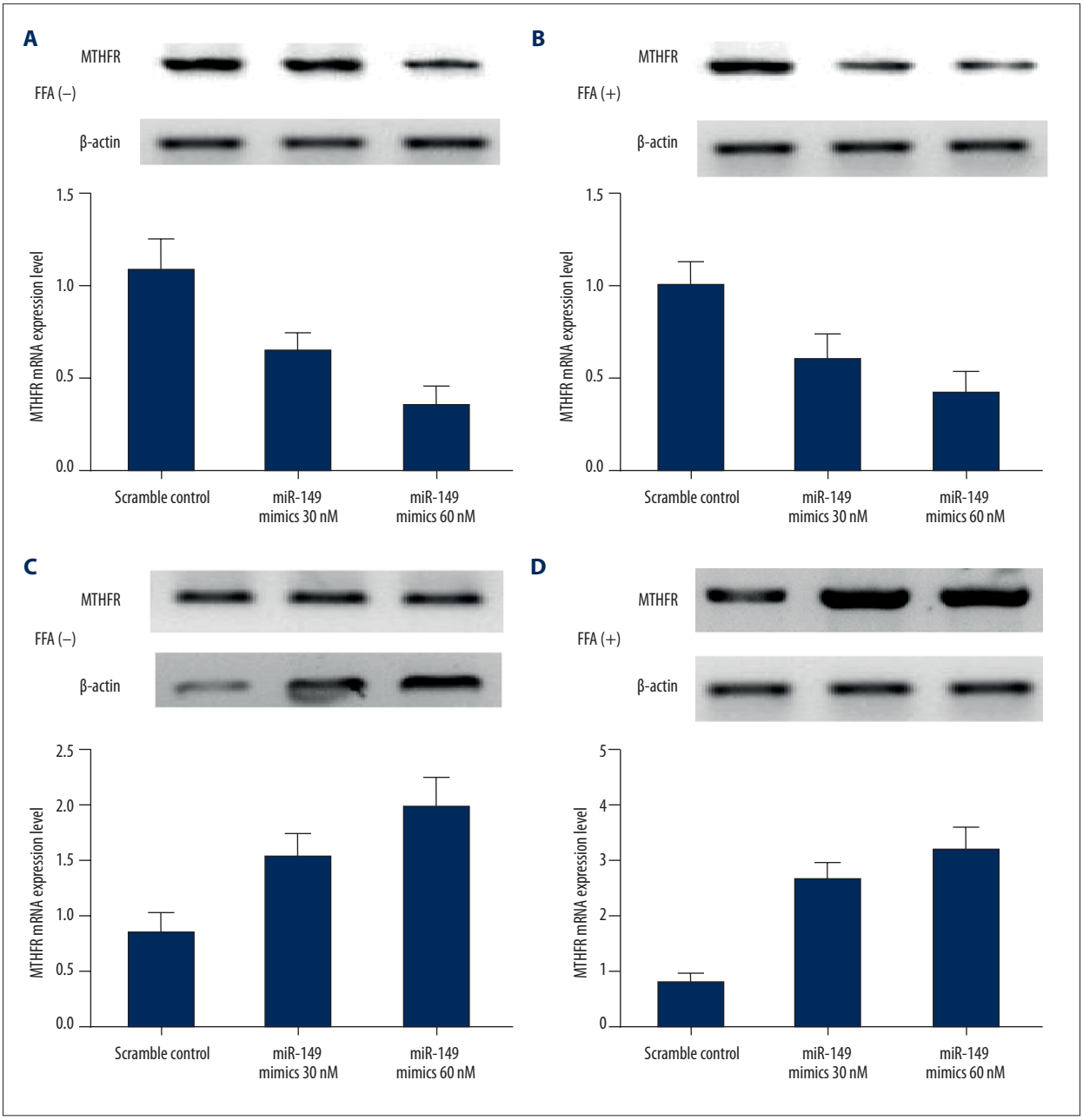

Figure 5. miR-149 attenuated the expression of MTHFR in hepatocytes genotyped as CC treated with or without FFA. (**A**) MTHFR level of cells treated without FFA transfected with 30 and 60 nM miR-149 mimics; (**B**) MTHFR level of cells treated with FFA transfected with 30 and 60 nM miR-149 mimics; (**C**) MTHFR level of cells treated without FFA transfected with 30 and 60 nM miR-149 inhibitors; (**D**) MTHFR level of cells treated with FFA transfected with 30 and 60 nM miR-149 inhibitors.

substantially related to NASH risks [29]. Nevertheless, in a Brazilian population, polymorphisms of A1298C and MTHFR C677T were not regarded as possible genetic risk factors for the onset of NAFLD [30]. The findings of Serin et al. also showed that in their Turkish cohort study that polymorphism of MTHFR C677T was not likely to be related to the progression of non-alcoholic steatosis to NASH [31]. The variants of MTHFR gene have been shown to be able to decrease the level of folate, the enzyme activity of MTHFR [32]. Kasapoglu B et al. suggested that homozygote mutations of A1298C and MTHFR C677T are positively related to the elevated concentrations of serum Hcy in NAFLD patients [33].

Some earlier reports have indicated the correlation between hepatic cancers and SNPs on miRNAs, including miR-149c (rs2292832) implicated in the reduction of cancer risk, while

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miR-106b-25 (rs999885) and miR-101-1 (rs7536540) were identified to be associated with the elevated risk of hepatic cancer [34,35]. As another pre-miRNA SNP, rs2292832C/T polymorphism in pre-mir-149 has been investigated in various cancers. Kim et al. showed that the C allele of hsa-mir-149 may play a protective role in male stomach cancer whereas the genotypes of mir-149 CT and CT/CC were related to a reduced risk of hepatic cancer [34]. Regardless of these results, rs2292832 was observed not associated with other malignancies, including head and neck squamous cell cancer, colorectal cancer, and lung cancer [36]. In the present study, we performed real-time PCR to detect the effect of FFA on the expression of miR-149 and MTHFR in hepatocytes genotyped as TT or CC, and revealed that miR-149 in hepatocytes genotyped as TT treat with FFA was evidently overexpressed, while MTHFR level was obviously downregulated related compared to the control. Whereas, miR-149 and MTHFR levels in hepatocytes genotyped as CC treated with FFA were comparable to the control. In this study, we applied Western blot analysis and real-time PCR to confirm the regulatory relationship between miR-149 and MTHFR, and the interaction between FFA and MTHFR levels in hepatocytes genotyped as TT or CC treated with or without FFA. We found that in hepatocytes genotyped as TT treated with or without FFA, MTHFR mRNA and protein levels in cells (genotyped as TT) treated with or without FFA was much higher following introduction of 30 nM miR-149 mimic compared to the control, and even higher subsequent to transfect with 60 nM miR-149 mimic compared to the control and 30 nM miR-149 mimic treatment. Whereas, MTHFR level in cells treated with or without FFA was significantly higher when transfection with 30 nM miR-149 inhibitor compared to the control, and even higher after transfection with 60 nM miR-149 inhibitor compared to the control and 30 nM miR-149 inhibitor group. In hepatocytes genotyped as CC treated with or without FFA, the trend of miR-149 caused an inhibition of expression of MTHFR that was similar with that in hepatocytes genotyped as TT treated with or without FFA.

Our study had some limitations. First, NAFLD and control participants were from just one hospital, which may trigger an inclusion deviation. Furthermore, the distribution of genotypes of rs2292832 was not verify via the Hardy Weinberg equilibrium in the controls, which indicated that our participants may not represent the average. Second, our study was conducted in a relatively small sample size, which may substantially decrease the power of the statistical analysis. Third, we used a cell model of NAFLD in this study, and further studies in animal models of NAFLD are warranted to confirm the observations of this study.

Conclusions

Our findings indicated that polymorphism of rs2292832 was related to the risk of NAFLD in a Chinese population. Additional studies should be conducted in more ethnic groups, and larger sample sizes should be required in future studies.

Conflict of interest

Authors report no conflict of interest.

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