Wang et al.: METTL14 promotes the progression of NALFD

Silencing METTL14 alleviates liver injury in non-alcoholic fatty liver disease by regulating mitochondrial homeostasis

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ABSTRACT

Mitochondrial dysfunction is an important pathogenic factor in non-alcoholic fatty liver disease (NAFLD). Methyltransferase-like 14 (METTL14) has been implicated in mitochondrial fission processes. This research aimed to investigate the mechanism of METTL14 in the mitochondrial function of NAFLD. We first established NAFLD mouse models and cell models, recording body and liver weights and examining pathological changes in liver tissues. Subsequently, serum levels of liver function indices (aspartate aminotransferase [AST], alanine aminotransferase [ALT], total cholesterol [TC], and triglycerides [TG]), inflammatory markers (tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6, and IL-1β), and mitochondrial dysfunction indicators (fission 1 protein [Fis1], dynamin-related protein 1 [Drp1], mitofusin 2 [Mfn2], SID1 transmembrane family member 2 [SIDT2], and mitochondrial membrane potential [MMP]) in the liver and cells were evaluated. The N6-methyladenosine (m6A) modification level of primary microRNA (pri-miRNA) and m6A enrichment on pri-miR-34a were quantified. Co-immunoprecipitation and dual-luciferase reporter gene assays were utilized to validate gene interactions. Our findings revealed highly elevated METTL14 expression in NAFLD mouse and cell models. Silencing METTL14 reduced weight gain and mitigated adverse liver function indices, inflammation, hepatic steatosis, and structural damage in NAFLD mice. It also led to a decrease in Fis1/Drp1 levels and an increase in MMP/Mfn2 in the liver and cells. Moreover, METTL14 increased the m6A level, promoting the binding of DGCR8 to pri-miR-34a, which enhanced miR-34a-5p expression. Databases and dual-luciferase reporter gene assays indicated that miR-34a-5p could suppress SIDT2 expression. The overexpression of miR-34a-5p or inhibition of SIDT2 expression negated the alleviative effects of METTL14 silencing on mitochondrial homeostasis imbalance. In conclusion, METTL14, through m6A modification, modulates the miR-34a-5p/SIDT2 axis, impairing mitochondrial homeostasis in NAFLD.
**Keywords:** Non-alcoholic fatty liver disease (NAFLD); methyltransferase-like 14 (METTL14); mitochondrial homeostasis; imbalance; N6-methyladenosine (m6A) modification; miR-34a-5p.
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a prevalent chronic liver disease with a global incidence of 32.4%. This condition can cause liver fibrosis, cirrhosis, and non-alcoholic steatohepatitis (NASH) [1]. The primary manifestations of NAFLD are liver fat accumulation and hepatic steatosis [2]. Insulin resistance leads to the occurrence of metabolic syndrome, which is a potential risk factor for NAFLD [3]. Currently, pharmacological treatments like vitamin E and pioglitazone are commonly prescribed for NAFLD. However, these treatments have limitations. For instance, vitamin E is not sufficiently effective against fibrosis, while pioglitazone can induce side effects such as weight gain and bone loss. Additionally, both treatments can increase the risks of bleeding and tumorigenesis [4]. An imbalance in mitochondrial homeostasis can lead to changes in mitochondrial number, morphology, and function, which may subsequently result in the occurrence of various diseases [5]. More importantly, mitochondrial dysfunction is known to exacerbate NAFLD [6]. In light of these findings, we conducted experiments to investigate the underlying mechanisms of the imbalance of mitochondrial homeostasis in NAFLD, aiming to provide additional therapeutic options for NAFLD patients.

RNA regulates gene expression through a variety of chemical modifications, among which N6-methyladenosine (m6A) is the most prevalent one. The methyltransferase like 3 (METTL3)/methyltransferase like 14 (METTL14) complex can incorporate m6A onto the messenger RNA (mRNA). This modification regulates the mRNA stability, splicing, processing, and nuclear export, and promotes the initiation of the mRNA translation process [7, 8]. METTL14, a common m6A methyltransferase, interacts with METTL3 to form a stable METTL3-METTL14 complex, which catalyzes the formation of m6A [9]. An overexpression of METTL14 induces the m6A modification, resulting in steatosis and hepatic fat accumulation [10]. Moreover, the activation of m6A methylation contributes to
mitochondrial redox imbalance and an increase in reactive oxygen species (ROS) production [11]. Therefore, m6A methylation plays a significant role in regulating mitochondrial homeostasis. Notably, several studies have reported elevated levels of METTL14 and m6A modification in NAFLD patients [12, 13]. These findings suggest a connection between m6A modification and NAFLD, as well as mitochondrial homeostasis imbalance. However, the regulatory mechanism through which m6A modification affects NAFLD by regulating mitochondrial homeostasis has not yet been fully elucidated.

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of protein-coding genes [14]. METTL14 catalyzes the modification of primary miRNAs (pri-miRNAs) with m6A, which promotes their recognition by DiGeorge syndrome critical region 8 (DGCR8). This interaction subsequently increases the expression of mature miRNA [15]. The dysregulation of miRNAs leads to excessive ROS production, heightened endoplasmic reticulum (ER) stress, the activation of the unfolded protein response, and thus impairment of mitochondrial function [16]. For example, overexpression of the miR-21-5p sequence causes metabolic changes and increases mitochondrial respiration in H9C2 cells [17]. Conversely, the knockdown of mitochondrial miR-1285 sequence alleviates mitochondrial respiratory dysfunction, adenosine triphosphate (ATP) deficiency, mitochondrial membrane potential (MMP) reduction, and the accumulation of mitochondrial ROS in pig jejunal epithelial cells [18]. Notably, the downregulation of the miR-34a-5p sequence mitigates NAFLD-associated fibrosis through the inhibition of hepatic stellate cells activation [19]. Additionally, the downregulation of miR-34a-5p sequence in the liver attenuates mitophagy, alleviates mitochondrial dysfunction, and repairs oxidative liver injury [20]. In our study, we further investigated the mechanism of miR-34a-5p in NAFLD, particularly its impact on regulating mitochondrial dysfunction.
The systemic RNA interference defective-1 (SID1) transmembrane family member 2 (SIDT2) is a highly glycosylated multichannel lysosomal transmembrane protein essential for maintaining the normal morphology of lysosomes [21]. The deletion of SIDT2 can lead to lipid droplets accumulation in the liver and it can hinder the maturation of autophagy-lysosomes in hepatocytes, resulting in failed breakdown of lipids into fatty acids in lysosomes, leading to the occurrence of NAFLD [22]. Moreover, silencing SIDT2 disrupts the regular mitochondrial fission and fusion processes, leading to mitochondrial dysfunction [23]. While some miRNAs have been identified as targeting SIDT2 in the context of liver diseases and cancer [24, 25], no studies have yet reported on the miR-34a-5p-mediated regulation of SIDT2. The mechanism through which m6A modification influences NAFLD by regulating mitochondrial homeostasis imbalance has not been fully explored. In this study, we reported that METTL14 exacerbates mitochondrial homeostasis imbalance, thereby promoting NAFLD. This effect is achieved by regulating the m6A modification of miR-34a, which in turn regulates SIDT2. Our findings provide new insights into potential treatment strategies for NAFLD.

MATERIALS AND METHODS

Experimental animal procedures
A total of 48 male C57BL/6J mice, aged 7-8 weeks and weighing between 18-22 g, were procured from Moslaite Biotechnology Co., Ltd (Hangzhou, China) (SYKX Zhejiang 2022-0032). In line with experimental animal raising standards, the mice were housed at 23°C with a 50% relative humidity and with free access to water and food for one week before the experiment. Subsequently, 12 mice were randomly selected and fed with a standard diet (LAD3001-10) to serve as the control group. The remaining mice were placed on a high-fat diet (TP 26300, Trophic Animal Feed High-tech Co., Ltd, Nantong, Jiangsu, China) for eight weeks to establish the NAFLD mouse model. The body weight variations were recorded
regularly. Following the high-fat diet regimen, the mice were administered lentiviral vectors injections containing either short hairpin (sh)-METTL14 or sh-negative control (NC) (GENCHEM, Shanghai, China) via the tail vein at a concentration of $1 \times 10^9$ TU/mL. For control purposes, an equivalent amount of saline was injected into mice through the tail vein. Eight weeks post-injection, mice were anesthetized using sodium pentobarbital (50 mg/kg). The fasting blood samples were drawn from the mice and centrifuged for 5 min at 3000 × g. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), and triglycerides (TG) were subsequently measured. Finally, euthanasia was carried out using an intraperitoneal injection of 150 mg/kg sodium pentobarbital [26] and the mice’s livers were excised and weighed. After weighing, the liver tissues of six randomly selected mice in each group were used for quantitative real-time polymerase chain reaction (qRT-PCR) and additional tests, while the liver tissues of the other six mice underwent hematoxylin-eosin (H&E) and other staining procedures.

**Hematoxylin-eosin, oil red O, and Masson staining assay**

Liver specimens were harvested, weighed, and promptly fixed in 4% paraformaldehyde. Following paraffin embedding, the liver tissues were sectioned to a thickness of 4 μm and deparaffinized. Oil red O staining was used to visualize the lipid deposition in the liver tissue, while Masson staining was employed to assess liver fibrosis.

**Enzyme-linked immunosorbent assay (ELISA)**

Liver tissues or serum samples were harvested from the mice. The levels of serum liver functional indices (AST and ALT) and blood lipid indices (TC and TG), as well as the expression levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β in the liver tissues were assessed. All measurements were conducted as per the steps instructed by the respective ELISA kits. The ELISA kits for AST (ab263882), ALT (ab282882), TC (ab285242), TNF-α (ab208348), IL-6 (ab222503), and IL-1β (ab197742) were sourced from Abcam (Cambridge, MA, USA). The ELISA kit for TG was procured from mlbio (ml095894,
Shanghai, China). The optical density of each well was recorded using a microplate reader set at a wavelength of 450 nm.

**Cell culture and transfection**

Human normal liver cells (L-02) were acquired from EDITGENE (EDLUCQ0106, Guangzhou, China). The cells were maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/L streptomycin. The cells were cultured in an incubator set at 37°C with 5% CO₂. To establish the NAFLD cell model, cells were treated with 1 mM fatty acid (comprising palmitic acid and oleic acid in a 1:2 concentration ratio) for 24 h. For the control group, an equivalent volume of culture medium was added. Plasmids, namely small interfering (si)-METTL14-1, si-METTL14-2, si-SIDT2-1, si-SIDT2-2, and si-NC, as well as miR-34a-5p-mimic and mimic-NC, were sourced from GenePharma (Shanghai, China). These plasmids or mimics were transfected into L-02 cells using the lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) and cultured for 48 h before the subsequent assays.

**Quantitative real-time polymerase chain reaction**

Total RNA was extracted from either mouse liver tissue or L-02 cells using the TRIzol reagent (Invitrogen). This RNA was subsequently reverse-transcribed into the complementary DNA (cDNA) utilizing a reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). For qRT-PCR, 1 μL of the cDNA was employed as per the instructions of the SYBR Green PCR Master Mix Kit (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 served as internal reference genes [27]. The relative expression levels of METTL14, SIDT2, pri-miR-34a, and miR-34a-5p were determined using the 2^{-ΔΔCt} method [28]. The sequences of the primers utilized are presented in Table 1.
**Western blot assay**

Total protein from mouse liver tissues and L-02 cells was extracted using the radioimmunoprecipitation assay lysis solution (Beyotime, Shanghai, China). Protein concentration was adjusted and quantified using the bicinchoninic acid assay kit. For protein separation, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis was employed. Subsequently, the separated proteins were transferred onto polyvinylidene fluoride membranes. These membranes were then blocked with skimmed milk for one h, followed by overnight incubation with primary antibodies against METTL14 (1:1000; ab300104; Abcam), Fis1 (1:500; AB_1950286; GeneTex, Irvine, CA, USA), Drp1 (1:1000; ab184247; Abcam), Mfn2 (ab50838; Abcam), SIDT2 (ab67299; Abcam), and GAPDH (1:1000; ab226408; Abcam). A one-hour incubation with the secondary antibody (1:2000; ab205718; Abcam) preceded visualization through enhanced chemiluminescence. Gray value analysis was performed using Image Lab software (Bio-Rad, Hercules, CA, USA).

**5,5,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolycarbocyanine iodide (JC-1) staining assay**

MMP was assessed using the JC-1 kit (Beyotime). Mouse liver tissues were minced, homogenized, and subjected to centrifugation at 1000 × g for 10 min at 4°C. The resultant supernatant was obtained and further centrifuged for 15 min. Following this, the supernatant was discarded, and the precipitate was diluted with 1 mL of buffer to produce a single-cell suspension for subsequent assays. Both this suspension and the L-02 cells were incubated in the dark at 37°C in an atmosphere of 5% CO2 and subsequently stained with JC-1 solution for 30 min. The cells were observed and photographed under a fluorescence microscope. The fluorescence intensity of JC-1 was quantitatively analyzed with the Image Pro Plus software.

**Quantification of m6A RNA methylation**

Total RNA was extracted from mouse liver tissue and L-02 cells using the TRIzol reagent. The quality of the extracted RNA was assessed using NanoDrop (Thermo Fisher, Waltham,
MA, USA) and further verified by 1% agarose gel electrophoresis. The levels of m6A in liver tissues and L-02 cells were quantified using the EpiQuik m6A RNA methylation quantification kit (Colorimetric, P-9005-48, Epigentek, Farmingdale, NY, USA). Absorbance was measured at a wavelength of 450 nm, and the relative m6A quantity was determined based on the standard curve.

**RNA immunoprecipitation (RIP) assay**

The RIP assay was performed using the Magna RIP kit (Millipore, Billerica, MA, USA). Both tissues and cells were lysed with RIP lysis buffer (Millipore) at 4°C using destructive ultrasonication. Endogenous DGCR8 was immunoprecipitated with the DGCR8 antibody (1:60; ab191875; Abcam) during an overnight incubation at 4°C. After the extraction of RNAs, the levels of pri-miR-34a were analyzed by qRT-PCR.

For the m6A RNA binding assay, RNAs were extracted from tissues and cells and treated with DNase I, followed by sonication to fragment the RNAs. The m6A antibody (ab264408; Abcam) was bound to magnetic beads, and RNA fragments were subsequently immunoprecipitated using the RIP assay. After a 1.5-hour treatment of the magnetic beads with proteinase K, RNAs were extracted, and the enrichment of pri-miR-34a was analyzed using qRT-PCR.

**Bioinformatics**

The microRNA Target Prediction Database (miRDB) ([http://mirdb.org/](http://mirdb.org/)) [29], TargetScan7.2 ([http://www.targetscan.org/vert_72/](http://www.targetscan.org/vert_72/)) [30], and miRWalk ([http://mirwalk.umm.uni-heidelberg.de/](http://mirwalk.umm.uni-heidelberg.de/)) [31] were used to predict the downstream target genes of miR-34a-5p. The TargetScan7.2 database was used to predict the binding site between miR-34a-5p and SIDT2.

**Dual-luciferase reporter gene assay**

The SIDT2 wild-type (WT) was constructed by amplifying the SIDT2 3'-UTR fragment that contains the miR-34a-5p binding site. The SIDT2 mutant type (MUT) was constructed by
site-directed mutagenesis. Both the SIDT2-WT and SIDT2-MUT sequences were then cloned into the pGL vector. These constructs were co-transfected into L-02 cells along with either the miR-34a-5p-mimic or mimic-NC using the lipofectamine 2000 transfection reagent. After 48 h of culture, the relative activity of luciferase was determined using the dual-luciferase kit (Beyotime).

**Ethical statement**

The animal experiment received approved from the animal Ethics Committee of Hunan Aerospace Hospital (Approval number: HNHTYY20221108LLSH-025-01). All experimental procedures involving animals were performed in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals [32].

**Statistical analysis**

Data analyses were conducted using the GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA). All measurement data are presented as mean ± standard deviation (SD). Pairwise data comparisons were assessed using the t-test, while multigroup data comparisons were evaluated using one-way or two-way analysis of variance (ANOVA), followed by post-hoc Tukey's multiple comparison test. A P value of < 0.05 was considered statistically significant.

**RESULTS**

**METTL14 is highly expressed in NAFLD mice and METTL14 silencing alleviates liver injury in NAFLD mice**

The mouse model of NAFLD was established using a high-fat diet to investigate the significance of METTL14 in NAFLD. After eight weeks on this diet, METTL14 expression was inhibited through a tail vein injection of lentivirus-packaged sh-METTL14. The body weight of mice was recorded regularly during the feeding process. It was found that the body weight of NAFLD mice began to increase significantly after the 4th week of high-fat diet feeding, with a significant increase in the weight of the liver also being observed (P < 0.05,
Concurrently, METTL14 was abundantly expressed in the NAFLD mice ($P < 0.05$, Figure 1C-D), accompanied by significantly elevated serum levels of AST, ALT, TC, and TG ($P < 0.05$, Figure 1E-H). Histological examination using the H&E staining revealed significant pathological alterations in the liver tissue (Figure 1I), along with significant increases in the TNF-α, IL-6, and IL-1β levels ($P < 0.05$, Figure 1J-L). A significant increase in the number of lipid droplets accumulated in the NAFLD liver was noted (Figure 1M), and Masson staining highlighted fibrotic changes in the liver of NAFLD mice (Figure 1N). Upon METTL14 silencing ($P < 0.05$, Figure 1C-1D), the body weight of the mice was significantly reduced starting from the 12th week ($P < 0.05$, Figure 1A-B). Compared to the NAFLD mice group, METTL14-silenced mice exhibited decreased levels of AST, ALT, TC, and TG ($P < 0.05$, Figure 1E-H). Their liver pathological changes showed significant improvements (Figure 1I), with reduced levels of TNF-α, IL-6, and IL-1β ($P < 0.05$, Figure 1J-L). Furthermore, reductions in the number of lipid droplets in the liver were observed (Figure 1M), and the degree of liver fibrosis was mitigated (Figure 1N). These results suggested that silencing METTL14 effectively slowed down the body weight gain, and alleviated liver injury, inflammation, steatosis, and tissue structural damage induced by a high-fat diet.

**METTL14 silencing alleviates the imbalance of mitochondrial homeostasis in NAFLD mice**

Mitochondrial homeostasis imbalance is the key cause of NAFLD [33, 34]. Previous studies have shown that METTL14-mediated m6A modification can promote mitochondrial fission [35]. Based on these findings, we speculated that METTL14 may improve liver injury in NAFLD mice by regulating mitochondrial homeostasis. The detection results of NAFLD mice revealed a decrease in MMP ($P < 0.05$, Figure 2A), an increase in the expression levels of mitochondrial fission proteins, namely mitochondrial fission 1 protein (Fis1) and dynamin-related protein 1 (Drp1), and a decrease in the fusion protein mitofusin 2 (Mfn2) expression ($P < 0.05$, Figure 2B). After METTL14 silencing, there was an increase in MMP
(P < 0.05, Figure 2A), a decrease in Fis1 and Drp1 expression levels, and an increase in Mfn2 expression (P < 0.05, Figure 2B). These results verified our speculation at the preliminary level.

**METTL14 silencing alleviates the imbalance of mitochondrial homeostasis in L-02 cells**

We next strived to verify that silencing METTL14 can alleviate mitochondrial homeostasis imbalance in NAFLD through in vitro assays. Fatty acid-treated L-02 cells were used to establish a cell model of NAFLD. After the fatty acid treatment, the results revealed an increase in METTL14 (P < 0.05, Figure 3A-B), a decrease of MMP (P < 0.05, Figure 3C), an increase in the levels of mitochondrial fission proteins Fis1 and Drp1, and a decrease in the fusion protein Mfn2 levels (P < 0.05, Figure 3D). To suppress the METTL14 expression, si-METTL14 was transfected into L-02 cells, which led to significant inhibition (P < 0.05, Figure 3A-B). Following this treatment, an increase of MMP was observed (P < 0.05, Figure 3C), Fis1 and Drp1 levels decreased, and Mfn2 levels increased (P < 0.05, Figure 3D). These results indicate that the mitochondrial homeostasis imbalance occurred in the NAFLD cell model, and that the inhibition of METTL14 expression improved the mitochondrial homeostasis.

**METTL14-mediated m6A modification promotes the DGCR8 binding to pri-miR-34a and increases the expression of mature miR-34a-5p**

Further investigation was conducted into the specific mechanism through which METTL14 affects mitochondrial homeostasis. As a methyltransferase, METTL14 catalyzes m6A modification to promote miRNA maturation [36]. We assessed the expression of m6A in liver tissues of NAFLD mice and in cell models. The detection results showed that m6A levels were highly elevated. When METTL14 was inhibited, m6A levels decreased significantly (P < 0.05, Figure 4A-B), suggesting the role of METTL14 in modulating m6A methylation in NAFLD. It has been reported that METTL14 can promote the m6A modification of pri-miR-34a, thereby promoting the expression of mature miR-34a-5p [37].
Given that the miR-34a-5p is highly expressed in NAFLD [19], we speculated that METTL14 might regulate the expression of miR-34a-5p in both the NAFLD animal and cell models. Subsequent to METTL14 expression inhibition, the level of pri-miR-34a bound to DGCR8 was significantly decreased, coupled with a significant decrease in the level of m6A-modified pri-miR-34a ($P < 0.05$, Figure 4C-F). Moreover, upon evaluating miR-34a-5p expression in mouse liver tissues and L-02 cells, we found that its levels were heightened in NAFLD. Inhibiting METTL14 expression led to a significant decrease in miR-34a-5p levels ($P < 0.05$, Figure 4G-H). These results suggested that METTL14-mediated m6A modification could promote the binding of DGCR8 to pri-miR-34a, thereby increasing mature miR-34a-5p expression.

**The overexpression of miR-34a-5p partially reverses the alleviative effect of METTL14 silencing on mitochondrial homeostasis imbalance**

We further investigated whether METTL14 regulates mitochondrial homeostasis through the regulation of miR-34a-5p expression. L-02 cells were transfected with miR-34a-5p-mimic to induce the overexpression of miR-34a-5p ($P < 0.05$, Figure 5A). This was done in combination with si-METTL14-2, which exhibited superior transfection efficiency. Following the overexpression of miR-34a-5p, the MMP decreased ($P < 0.05$, Figure 5B). The protein levels of Fis1 and Drp1 increased, while the protein expression of Mfn2 significantly decreased ($P < 0.05$, Figure 5C). These results suggest that the overexpression of miR-34a-5p could partially reverse the alleviative effect of METTL14 silencing on the mitochondrial homeostasis imbalance.

**SIDT2 is identified as a downstream target gene of miR-34a-5p**

Next, to elucidate the downstream regulatory mechanism of miR-34a-5p, we employed online databases including TargetScan7.2, miRWalk, and miRDB to screen for potential target genes. Among the identified candidates, SIDT2 emerged as a common target (Figure 6A). Through literature review, we found that SIDT2 plays a role in lipid metabolism, and
its deletion significantly affects NAFLD progression [22, 38]. TargetScan7.2 prediction revealed that miR-34a-5p had a binding sequence with SIDT2 (Figure 6B), which was subsequently confirmed through the dual-luciferase reporter gene experiment \( (P < 0.05, \text{Figure } 6C) \). SIDT2 was poorly expressed in liver tissues of NAFLD mice and fatty acid-treated L-02 cells. However, its expression was significantly increased after METTL14 silencing \( (P < 0.05, \text{Figure } 6D-G) \). Moreover, cellular assays indicated that, after METTL14 silencing, the overexpression of miR-34a-5p led to a significant decrease in SIDT2 expression \( (P < 0.05, \text{Figure } 6F, 6G) \). These results suggest that SIDT2 acts as a downstream target gene of miR-34a-5p. Furthermore, within the context of NAFLD, miR-34a-5p appears capable of targeting and inhibiting SIDT2 expression.

**Inhibition of SIDT2 partially reverses the alleviative effect of METTL14 silencing on mitochondrial homeostasis imbalance**

Subsequently, we further sought to verify whether SIDT2 was involved in METTL14’s regulation of mitochondrial homeostasis. L-02 cells were transfected with si-SIDT2-1 and si-SIDT2-2 to suppress the SIDT2 expression \( (P < 0.05, \text{Figure } 7A-B) \). For combination experiments, we utilized si-METTL14-2 and si-SIDT2-1, both of which exhibited higher transfection efficiencies. Following the SIDT2 suppression, the MMP decreased \( (P < 0.05, \text{Figure } 7B) \). The protein levels of Fis1 and Drp1 increased, while the protein expression of Mfn2 significantly decreased \( (P < 0.05, \text{Figure } 7C) \). These results suggest that the inhibition of SIDT2 could partially reverse the alleviative effect of METTL14 silencing on the mitochondrial homeostasis imbalance.

**DISCUSSION**

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive accumulation of triglycerides, inflammation, injury, and apoptosis [39]. Mitochondrial dysfunction is one of the most significant features of NAFLD, and the significant suppression of mitochondrial parameters occurs in NAFLD patients [40]. Previous studies have focused on nuclear
receptors and compounds and mitochondrial metabolism-related enzymes as potential targets of mitochondrial dysfunction in NAFLD. However, the regulation of m6A modifying enzymes in mitochondria function has rarely been reported [41]. In this study, we found that 1) METTL14 catalyzed the m6A modification of pri-miR-34a to promote the binding of pri-miR-34a to DGCR8 to increase the expression of mature miR-34a-5p; 2) miR-34a-5p bound to SIDT2 mRNA and inhibited SIDT2 expression; 3) Inhibition of SIDT2 promoted the expression of mitochondrial fission protein Fis1 and Drp1 and inhibited the expression of mitochondrial fusion protein Mfn2, leading to imbalance of mitochondrial homeostasis and aggravation of NAFLD (Figure 8).

Moreover, mitochondrial dysfunction is characterized by increased reactive oxygen species, reduced MMP, increased mitochondrial fission proteins Fis1 and Drp1, decreased mitochondrial fusion protein Mfn2, and mitochondrial membrane damage, which leads to hepatic lipid accumulation in NAFLD [34]. Aberrant upregulation of METTL14 and m6A resulted in hepatic lipolysis suppression, adipose expansion, and metabolic disorders [42]. METTL14 enhances m6A modification of Fis1, enhances its expression, and promotes mitochondrial fission [35]. Therefore, we hypothesized that METTL14 could participate in NAFLD by mediating m6A modification to disrupt mitochondrial homeostasis. Serum levels of AST, ALT, TC, and TG are common diagnostic indicators of steatosis, liver fibrosis, liver cirrhosis, and NAFLD [43]. Overexpression of METTL14 increased the levels of AST, ALT, TG, TC, pro-inflammatory factors, and lipid accumulation, promoting chronic inflammation and cell apoptosis, which is a case in NAFLD [10]. In our study, it was found that METTL14 was overexpressed and serum levels of AST, ALT, TC, TG, and pro-inflammatory factors TNF-α, IL-6, and IL-1β were significantly increased in NAFLD mice. Silencing METTL14 counteracted the increases of these indicators and ameliorated liver inflammation and liver tissue damage in mice. Furthermore, MMP was decreased, the expression of mitochondrial
fission proteins Fis1 and Drp1 was increased, and the expression of fusion protein Mfn2 was decreased in NAFLD mouse and in vitro cell models. Silencing METTL14 reversed the above changes, suggesting that inhibition of METTL14 expression maintained mitochondrial homeostasis and reduced liver injury after NAFLD.

Methyltransferase catalyzes m6A modification, and promotes the binding between DGCR8 and pri-miRNA, leading to miRNA maturation [44]. Previous reports demonstrated that METTL14-mediated m6A modification improves the binding of pri-miR-375 to DGCR8, conversion of pri-miR-375 into mature miR-375, and induction of neuronal apoptosis in the spinal cord [45]. METTL14-mediated m6a methylation positively regulates the maturation of miR-34 and promotes the expression of miR-34a-5p [37]. Our experiments confirmed that inhibition of METTL14 reduced the level of pri-miR-34a bound to DGCR8 and decreased the expression of miR-34a-5p, suggesting that METTL14-mediated m6A modification could promote the binding of DGCR8 to pri-miR-34a to increase the expression of mature miR-34a-5p. Moreover, a previous study has shown that miR-34a-5p is overexpressed in NAFLD and induces glucose and lipogenesis in hepatocytes [46]. Overexpression of miR-34a-5p mediates mitophagy damage, causing the accumulation of dysfunctional mitochondria and leading to oxidative liver injury [20]. After overexpression of miR-34a-5p, MMP was decreased, the levels of Fis1 and Drp1 proteins were increased, and Mfn2 protein was significantly decreased, suggesting that overexpression of miR-34a-5p counteracted the ameliorative effect of METTL14 silencing on mitochondrial homeostasis.

Database analysis showed that SIDT2 may be a downstream target of miR-34a-5p. In SIDT2-deficiency mice, a large accumulation of lipid droplets was found in the liver tissues, as well as elevated ALT and AST, suggesting hepatocellular injury occurs [22]. In addition, SIDT2-deficiency mice developed ER stress and unfolded protein stress, which aggravated NAFLD [38]. SIDT2 deletion impairs the fusion of autophagosomes and lysosomes, leading to a
decrease in the number of acidic lysosomes and the destruction of lysosomal function, which in turn causes an imbalance in mitochondrial homeostasis [47, 48]. The expression of SIDT2 was downregulated in NAFLD mouse liver tissues and L-02 cells, and silencing METTL14 significantly increased SIDT2. Under these conditions, we overexpressed miR-34a-5p and found that SIDT2 expression was reduced, suggesting that METTL14 promoted the expression of miR-34a-5p to inhibit the expression of SIDT2. After SIDT2 expression was inhibited, MMP was decreased, the expression levels of Fis1 and Drp1 proteins were increased, and Mfn2 protein in the cells was decreased, suggesting that inhibition of SIDT2 counteracted the alleviative effect of METTL14 on imbalance of mitochondrial homeostasis.

Our study still has some limitations. First of all, although METTL14 can catalyze m6A modification to promote the maturation of multiple miRNAs, our study only explored miR-34a-5p and did not explore the effects of other miRNAs in mitochondrial homeostasis and NAFLD. Secondly, except SIDT2, other downstream target genes of miR-34a-5p were not explored. Thirdly, other m6A-modifying enzymes were not studied. Finally, we only explored the m6A modification of miR-34a mediated by METTL14, but did not explore whether there was m6A modification on the mRNA of SIDT2 and whether it played a role in NAFLD, and whether other genes were regulated by m6A modification to be involved in NAFLD. In the future, we will select other miRNAs and other target genes of miRNAs for further study, continue to investigate the function of other m6A modifying enzymes such as METTL3 in mitochondrial homeostasis imbalance and NAFLD, improve the exploration of the regulatory mechanism of SIDT2 in NAFLD, explore the genome-wide/transcriptome-wide epitranscriptomic profiling of miRNA and mRNA, and determine whether other miRNAs or mRNAs are involved in epitranscriptomic regulation of NAFLD.
CONCLUSION

In summary, our study revealed that in NAFLD, METTL14 is upregulated and subsequently facilitates the binding of pri-miR-34a to DGCR8, leading to an increase mature miR-34a-5p expression. This elevated miR-34a-5p then suppresses SIDT2 expression, resulting in an imbalance in mitochondrial homeostasis that aggravates NAFLD. Our study revealed a novel mechanism of METTL14 in NAFLD, offering potential targets and new directions for disease treatment.

Data availability

The datasets generated or analyzed during the current study are available from the corresponding author upon reasonable request.
REFERENCES


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### TABLE 1. qRT-PCR sequences of utilized primers

<table>
<thead>
<tr>
<th>Forward primer (5′-3′)</th>
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<tr>
<td>mmu-METTL14</td>
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<td>mmu-miR-34a-5p</td>
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**qRT-PCR:** Quantitative real-time polymerase chain reaction; mmu: Mus musculus; METTL14: Methyltransferase like 14; SIDT2: SID1 transmembrane family member 2; pri: primary; miR: MicroRNA; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; hsa: Homo sapiens.
FIGURE 1. Elevated METTL14 expression in NAFLD mice and its attenuation through silencing. The NAFLD mouse model was established by high-fat diet. After 8 weeks of high-fat diet feeding, the expression of METTL14 was silenced by a tail vein injection of lentivirus packaged with sh-METTL14 (with sh-NC as control). (A) Regular recording of the mice's body weight throughout the feeding period. (B) Liver weight recorded at the conclusion of feeding regimen. (C) Western blot assay showing METTL14 protein expression in liver tissue. (D) qRT-PCR analysis of METTL14 mRNA levels in liver tissues. (E-H) ELISA measurements of serum levels of AST (E), ALT (F), TC
(G), and TG (H). (I) H&E staining assay revealing the pathological alterations in mouse liver tissues. (J-L) ELISA measurements of TNF-α (J), IL-6 (K), and IL-1β (L) levels in mouse liver tissues. (M) Lipid accumulation in the liver visualized using Oil Red O staining. (N): Liver fibrosis assessed by Masson staining. For panels A-B and E-H the number of mice was \( n = 12 \); for panels C-D and I-N the number of mice was \( n = 6 \). Data are presented as mean ± standard deviation. Data in panel A were analyzed using the two-way ANOVA test, while data in panels B-L were evaluated with one-way ANOVA, followed by Tukey’s post-hoc test. In A: * indicates comparison with the control group, # indicates comparison with the sh-NC group, both at \( P < 0.05 \). In B-L: # signifies a \( P < 0.05 \).

METTL14: Methyltransferase like 14; NAFLD: Non-alcoholic fatty liver disease; sh: Short hairpin RNA; NC: Negative control; qRT-PCR: Quantitative real-time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TC: Total cholesterol; TG: Triglycerides; H&E: Hematoxylin-eosin; TNF-α: Tumor necrosis factor-alpha; IL: Interleukin; ANOVA: Analysis of variance; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; mRNA: Messenger RNA.
FIGURE 2. METTL14 silencing alleviates mitochondrial homeostasis imbalance in NAFLD mice. The NAFLD mouse model was established by high-fat diet. After 8 weeks of high-fat diet feeding, the expression of METTL14 was silenced by a tail vein injection of lentivirus packaged with sh-METTL14 (with sh-NC as control). (A) The JC-1 staining assay employed to assess mitochondrial membrane potential in mouse liver tissue. (B) Western blotting assay used to detect the protein expression levels of Fis1, Drp1, and Mfn2 in liver tissue. For each panel the number of mice was \( n = 6 \). Data are presented as mean ± standard deviation. Data were analyzed using the one-way ANOVA test, followed by Tukey’s post-hoc test. \(^*P < 0.05\). METTL14: Methyltransferase like 14; NAFLD: Non-alcoholic fatty liver disease; sh: Short hairpin RNA; NC: Negative control; JC-1: 5,5,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide staining assay; Fis1: Fission 1 protein; Drp1: Dynamin-related protein 1; Mfn2: Mitofusin 2; ANOVA: Analysis of variance; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
FIGURE 3. METTL14 silencing alleviates mitochondrial homeostasis imbalance in L-02 cells.
L-02 cells were treated with fatty acids to establish the NAFLD cell model, and si-METTL14-1 and si-METTL14-2 (si-NC as control group) were transfected into the L-02 cells to reduce the expression of METTL14. (A, B) qRT-PCR (A) and western blot assay (B) used to assess METTL14 expression in the cells. (C) JC-1 staining assay employed to evaluate mitochondrial membrane potential. (D) Western blotting assay used to detect the protein levels of Fis1, Drp1, and Mfn2 in the cells. All cell experiments were performed three times independently. Data are presented as mean ± standard deviation. Data were analyzed using the one-way ANOVA test, followed by Tukey’s post-hoc test. *P < 0.05. METTL14: Methyltransferase like 14; L-02 cells: Human normal liver cells; NAFLD: Non-alcoholic fatty liver disease; si: Small interfering; NC: Negative control; qRT-PCR: Quantitative
real-time polymerase chain reaction; JC-1: 5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazoylcarbocyanine iodide staining assay; Fis1: Fission 1 protein; Drp1: Dynamin-related protein 1; Mfn2: Mitofusin 2; ANOVA: Analysis of variance; mRNA: Messenger RNA; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
FIGURE 4. METTL14-mediated m6A modification promotes DGCR8 binding to pri-miR-34a and increases the expression of mature miR-34a-5p. Samples of liver tissues from NAFLD mice and L-02 cells were collected. (A, B) RNA m6A quantification employed to determine m6A levels in mouse liver tissues (A) and L-02 cells (B). (C) qRT-PCR utilized to measure the levels of pri-miR-34a bound to DGCR8 in mouse liver tissues. (D) qRT-PCR utilized to measure the levels of m6A-modified pri-miR-34a in liver tissues. (E) qRT-PCR utilized to measure the level of pri-miR-34a bound to DGCR8 in L-02 cells (F) qRT-PCR utilized to measure the levels of m6A-modified pri-miR-34a in L-02 cells. (G-H) qRT-PCR utilized to measure the expression of miR-34a-5p in mouse liver tissues (G) and in L-02 cells (H). For A, C, D, and G the number of mice was n = 6. Experiments for panels B, E, F, and H were performed three times independently. Data are presented as mean ± standard deviation. Data in panels A, B, G, and H were analyzed using the one-way ANOVA test. Data in panels C-F were analyzed using the two-way ANOVA test, followed by Tukey’s post-hoc test. *P < 0.05. METTL14: Methyltransferase like 14; m6A: N6-methyladenosine; DGCR8: DiGeorge syndrome critical region 8; pri: Primary; miR: MicroRNA; NAFLD: Non-alcoholic fatty liver disease; L-02: Human normal liver cells; qRT-PCR: Quantitative real-time polymerase chain reaction; ANOVA: Analysis of variance; sh: Short hairpin; NC: Negative control; IgG: Immunoglobulin G.
FIGURE 5. Overexpression of miR-34a-5p partially reverses the alleviative effect of METTL14 silencing on mitochondrial homeostasis imbalance. L-02 cells underwent transfection with miR-34a-5p-mimic (with NC-mimic as control). (A) qRT-PCR utilized to verify the transfection efficiency of miR-34a-5p-mimic. The combined experiment was conducted with si-METTL14-2, which displayed better silencing efficiency. (B) JC-1 staining assay utilized to assess the mitochondrial membrane potential. (C) Western blotting assay utilized to determine the protein levels of Fis1, Drp1, and Mfn2 in the cells. Cell experiments were performed three times independently. Data are presented as mean ± standard deviation. Data in panels A-F were analyzed using the one-way ANOVA test, followed by Tukey’s post-hoc test. *P < 0.05. miR: MicroRNA; METTL14: Methyltransferase like 14; L-02: Human normal liver cells; NC: Negative control; qRT-PCR: Quantitative real-time polymerase chain reaction; si: Small interfering; JC-1: 5,5,6,6’-tetrachloro-1,1‘,3,3’-tetrathylbenzimidazolylcarbocyanine iodide staining assay; Fis1: Fission 1 protein; Drp1: Dynamin-related protein 1; Mfn2: Mitofusin 2; ANOVA: Analysis of variance; NAFLD: NAFLD: Non-alcoholic fatty liver disease; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
FIGURE 6. Identification of SIDT2 as a downstream target gene of miR-34a-5p. (A) Online databases TargetScan7.2, miRWalk, and miRDB employed to predict potential downstream targets of miR-34a-5p. (B) TargetScan7.2 utilized to predict the binding sequence between miR-34a-5p and SIDT2. (C) A dual-luciferase reporter gene assay conducted to verify the relationship between miR-34a-5p and SIDT2. The liver tissues of NAFLD mice and L-02 cells were harvested. (D) qRT-PCR utilized to measure the mRNA expression of SIDT2 in mouse liver tissues. (E) Western blot assay utilized to determine the expression of SIDT2 protein in mouse liver tissue. (F) qRT-PCR utilized to measure the mRNA expression of SIDT2 in L-02 cells. (G) Western blot assay utilized to determine the expression of SIDT2 protein in L-02 cells. For panels C, F-G, the cell experiments were performed three times independently. For panels D-E the number of mice was n = 6. Data in panel C were analyzed using the two-way ANOVA test. Data in panels D-G were analyzed using the one-way ANOVA test.
ANOVA test, followed by Tukey’s post-hoc test. *P < 0.05. SIDT2: SID1 transmembrane family member 2; miR: MicroRNA; miRDB: microRNA Target Prediction Database; NAFLD: Non-alcoholic fatty liver disease; L-02: Human normal liver cells; qRT-PCR: Quantitative real-time polymerase chain reaction; mRNA: Messenger RNA; ANOVA: Analysis of variance; WT: Wild-type; hsa: Homo sapiens; MUT: Mutant type; NC: Negative control; sh: Short hairpin; METTL14: Methyltransferase like 14; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; si: Small interfering.
FIGURE 7. Inhibition of SIDT2 expression partially reverses the alleviative effect of METTL14 silencing on mitochondrial homeostasis imbalance. L-02 cells were transfected with si-SIDT2-1 and si-SIDT2-2 (with si-NC as control). (A) qRT-PCR utilized to verify the transfection efficiency. For combined experiments, si-METTL14-2 was paired with si-SIDT2-1 due to its higher transfection efficiency. (B) Western blot assay utilized to determine the protein levels of SIDT2 in the L-02 cells. (C) JC-1 staining assay employed to evaluate mitochondrial membrane potential. (D) Western blot assay utilized to determine the protein levels of Fis1, Drp1, and Mfn2 in the L-02 cells. All cell experiments were performed three times independently. Data are presented as mean ± standard deviation. Data in panels A-D were analyzed using the one-way ANOVA test, followed by Tukey’s
post-hoc test. *$P < 0.05$. SIDT2: SID1 transmembrane family member 2; METTL14: Methyltransferase like 14; L-02: Human normal liver cells; si: Small interfering; NC: Negative control; qRT-PCR: Quantitative real-time polymerase chain reaction; JC-1: 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazoylcarbocyanine iodide staining assay; Fis1: Fission 1 protein; Drp1: Dynamin-related protein 1; Mfn2: Mitofusin 2; ANOVA: Analysis of variance; mRNA; Messenger RNA; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NAFLD: Non-alcoholic fatty liver disease.
FIGURE 8. METTL14’s role in non-alcoholic fatty liver disease through mitochondrial homeostasis regulation. METTL14 upregulation catalyzes the m6A modification of pri-miR-34a, enhancing the binding of pri-miR-34a to DGCR8, which in turn increases the expression of mature miR-34a-5p. This miR-34a-5p then binds to SIDT2 mRNA, thereby suppressing SIDT2 expression. As a result, the expression of mitochondrial fission proteins Fis1 and Drp1 is upregulated, while the expression of the mitochondrial fusion protein Mfn2 is downregulated. This cascade contributes to an imbalance in mitochondrial homeostasis, aggravating NAFLD. METTL14: Methyltransferase like 14; m6A: N6-methyladenosine; pri: Primary; miR: MicroRNA; DGCR8: DiGeorge syndrome critical region 8; SIDT2: SID1 transmembrane family member 2; mRNA: Messenger RNA; Fis1: Fission 1 protein; Drp1: Dynamin-related protein 1; Mfn2: Mitofusin 2; NAFLD: Non-alcoholic fatty liver disease; pre: Precursor.