

Original Article

YTHDF2 influences hepatic fibrosis by regulating ferroptosis in hepatic stellate cells by mediating the expression of ACSL4 in an m⁶A-dependent manner

Wentao Liu*, Yuan He, Kunlun Chen, Jianwen Ye, Long Yu, Chuang Zhou, and Wenlong Zhai*

Department of Hepatobiliary and Pancreatic Surgery, the First Affiliated Hospital of Zhengzhou University, Zhengzhou 450000, China

*Correspondence address. Tel: +86-15137163440; E-mail: splwt@sina.com (W.L.) / Tel: +86-13937150977; E-mail: fcczhaiwl@zzu.edu.cn (W.Z.)

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Abstract

Hepatic fibrosis (HF) is an abnormal reparative response of the liver to chronic injury and is histologically reversible. In recent years, increasing interest has been given to changes in m⁶A in liver disease. In this study, we explore the role of the m⁶A-modified reading protein YTHDF2 in HF and its regulatory mechanism. The HF mouse model is generated through CCl₄ injection, and the cell model is via TGF-β stimulation. The liver tissues are subjected to hematoxylin-eosin, Masson, and α-SMA immunohistochemical staining. Reactive oxygen species (ROS) and iron levels are examined via relevant kits. Quantitative real-time PCR, immunofluorescence staining, and western blot analysis were conducted to measure the YTHDF2 and ACSL4 levels. RNA immunoprecipitation, methylated RNA immunoprecipitation, RNA pull-down, and polysome fractionation were performed to understand the regulatory mechanism by which YTHDF2 affects ACSL4. The results show that YTHDF2 is highly expressed after HF induction, and the inhibition of YTHDF2 reduces fibrosis as well as ROS and iron levels. *In vitro*, overexpression of YTHDF2 increases hepatic stellate cell activation, as well as ROS and iron levels, and this effect is blocked by the silencing of ACSL4. YTHDF2 acts as a regulator of ACSL4 expression and is involved in m⁶A modification. In addition, *in vivo* experiments indicate that overexpression of ACSL4 reverses the attenuating effect of YTHDF2 interference on HFs. Therefore, YTHDF2 mediates the expression of the ferroptosis marker protein ACSL4 in an m⁶A-dependent manner, thereby affecting HF.

Key words YTHDF2, ACSL4, hepatic fibrosis, m⁶A, ferroptosis

Introduction

Hepatic fibrosis (HF), an injury caused by liver disease, results in the development of scar tissue called fibrosis, resulting in impaired liver function, and has become a serious health problem worldwide [1]. HF is reversible, but when liver fibrosis progresses to the stage of cirrhosis, the prognosis is relatively poor. Thus, revealing the possible molecular events of HF remission is highly important for the development of new therapeutic strategies.

Ferroptosis is triggered when glutathione-dependent antioxidant defenses falter, leading to uncontrolled lipid peroxidation and ultimate cell death [2]. Intracellular iron level affects the development of liver disease and the production of cellular reactive oxygen species (ROS) as well as lipid peroxides [3]. HSCs play a key role in

the progression of HF by increasing the production and secretion of the extracellular matrix, whereas ferroptosis contributes to HSC activation and HF progression [4]. Acyl-CoA synthetase long-chain family member 4 (ACSL4) is involved in fatty acid metabolism and synthesis. ACSL4 has been reported to facilitate ferroptosis in tumor cells [5]. In addition, ACSL4 can increase fibrosis and promote ferroptosis in hepatocellular carcinoma (HCC) [6]. Livers lacking ACSL4 exhibit less fibrosis and proliferation, especially in the HCC model of toxic injury [6]. However, further studies are needed to assess whether ACSL4-mediated ferroptosis has a direct effect on HF.

N⁶-methyladenosine (m⁶A), considered one of the most common chemical modifications of mRNAs [7], is reversible, dynamically added by the m⁶A methyltransferase, and cleared away by the

demethylase [8]. In addition, m⁶A exerts multiple biological effects by being directly bound to and recognized by the “reader” proteins of m⁶A in mRNAs and influences these m⁶A-modified mRNAs in numerous ways, including their stability, translation, and output [9]. Recent findings indicate that m⁶A readers play a critical role in the development of liver diseases—for example, the m⁶A reading protein YTHDF3 mediates PRDX3 translation to alleviate HF [10]. YTHDF2, from the same family, regulates cystathionine-beta-synthase expression in an m⁶A-dependent manner in gastric cancer, indirectly causing changes in ACSL4 expression [11]. Moreover, YTHDF2 expression is upregulated in HCC and is considered to be a marker of poor prognosis in HCC patients [12]. Therefore, can YTHDF2 directly interfere with ACSL4 expression by regulating the m⁶A methylation of ACSL4, and does this mode interfere with ferroptosis level and affect HF level?

Here, we propose that YTHDF2 mediates ACSL4 expression in an m⁶A-dependent manner to influence ferroptosis and thus regulate HF.

Materials and Methods

Animal model

C57BL/6 mice (Six- to eight-week-old, weighing between 18 g and 22 g) were obtained from CAVENS (Changzhou, China). The study received approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. 2024-KY-0620-001). To induce the HF model, the mice were injected with carbon tetrachloride (CCl₄, 2 mL/kg; Sigma-Aldrich, St Louis, USA) peritoneally, whereas the mice in the sham group were injected with olive oil [10]. The adeno-associated viruses AAV9-si-YTHDF2/NC or AAV9-oe-ACSL4/NC were injected into HF model mice via the tail vein (1×10^{12} µg/mL).

Hematoxylin-eosin, Masson, and immunohistochemical staining

Tissues from mouse livers were removed and fixed in paraformaldehyde for 24 h. Subsequently, the tissues were dehydrated with gradient ethanol (Sinopharm, Shanghai, China), cleared with xylene (Sinopharm), and embedded in paraffin (carefully so as not to have bubbles). The 5-µm slices were then subjected to tissue staining. For hematoxylin-eosin (HE) staining, the sections were processed with hematoxylin for 10 min and eosin (Beyotime, Shanghai, China) for 1 min. For Masson staining, the slices were stained with a Masson's Trichrome stain kit (Solarbio, Beijing, China) according to the manufacturer's instructions. For immunohistochemical staining, the slices were heated via microwave radiation in sodium citrate buffer (Sinopharm) and cooled to room temperature. After the samples were shaken with PBS (Beyotime), the tissues were circled with a tissue pen (Beyotime) and incubated with a solution containing 3% hydrogen peroxide (Sinopharm) for 15 min. Then, BSA (Sigma-Aldrich) was added for blocking. Thirty minutes later, the slices were incubated with the primary antibody against α -SMA (ab7817, 1 µg/mL; Abcam, Cambridge, UK) for 18 h, and the IHC detection reagent (#8125; Cell Signaling Technology, Boston, USA) was added and incubated for 30 min. Finally, the DAB color solutions (Beyotime) were added, and the images were observed under a DMi1 microscope (Leica, Wetzlar, Germany).

Lipid ROS detection

BODIPY™ 665/676 (B3932; Thermo Fisher Scientific, Waltham,

USA) was used for detection. The reagent was added to LX2 cells or a liver tissue cell suspension. After incubation at 37°C for 30 min, PBS was applied to wash the sample. Finally, the nuclei were stained with DAPI solution (C1005; Beyotime) before visualization via the DMi1 microscope.

Iron content examination

The iron content in the liver and LX2 cells was examined with an iron assay (ab83366; Abcam). To be specific, first, a fresh set of standards was prepared. One hundred microlitres of the diluted standards was added to the standard wells, as was 5 µL of the iron reducer. The samples were collected, lysed, diluted with iron assay buffer, and then added to the sample wells (100 µL/well). Subsequently, 5 µL of iron reducer was added to each sample. The standards and samples were mixed and incubated at 37°C for half an hour. After that, a 100 µL iron probe containing the iron standard I/iron standard was added to each well and incubated for 1 h in the dark. The output was instantly read at 593 nm via a colorimetric microplate reader (Bio-Rad, Hercules, USA).

Quantitative real-time PCR (qRT-PCR) analysis

TRIzol reagent obtained from Beyotime was used to extract RNA from liver tissues as well as LX2 cells. The obtained RNA samples were subjected to qRT-PCR analysis using the One-Step qRT-PCR kit (Beyotime). The relative *YTHDF2* and *ACSL4* mRNA levels were calculated via the 2^{-ΔΔCT} method. *GAPDH* served as the control.

Cell culture and transfection

LX2 cells were cultured to 60% confluence before transfection. Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) was mixed with si-YTHDF2, si-ACSL4, or pc-YTHDF2 (RiboBio, Guangzhou, China), followed by incubation for 5 min at room temperature. The mixture was then added to LX2 cells and incubated for 48 h. The sequence information was as follows: si-YTHDF2: GGATGGATTAAACGATGATGA; si-ACSL4: GGCTGTAC TGCTATTCTTACT; and si-NC: AAGCTTCATAAGGCGCATAGC.

Western blot analysis

Using Beyotime RIPA lysis buffer (#P0013B), we obtained proteins from liver tissues and LX2 cells. The samples were subsequently centrifuged in a microcentrifuge at 4°C and spun at 13,780 *g* for 20 min. After that, the centrifuge tube was gently removed and placed on ice. Soon afterward, the supernatant was removed and transferred to a new tube, which was dropped onto ice already used for cooling, and the precipitate was discarded. The protein was quantified using a BCA kit (Thermo Fisher Scientific) and boiled for 5 min in a water bath. Equal amounts of protein and molecular weight markers (Beyotime) were subsequently separated via SDS-PAGE. After the activation of the PVDF membrane (Thermo Fisher Scientific) with methanol (Sinopharm), it was washed with transmembrane buffer, and the protein was subsequently transferred onto the membranes. The membranes were then blocked at room temperature for 1 h and incubated with the appropriately diluted primary antibody at 4°C overnight. Following washing with TBST buffer (Beyotime), the secondary antibody was applied to the above-mentioned membranes, and incubation was carried out at room temperature for 1 h. Chemiluminescence images were collected in the dark. The antibodies used were as follows: anti-YTHDF2 (#71283, 1/1000; Cell Signaling Technology), anti-ACSL4

(#sc-271800, 1/300; Santa Cruz Biotechnology, Shanghai, China), anti-Tubulin (5 µg/mL, ab44928; Abcam), 6× His Tag Polyclonal Antibody (#6XHIS-101AP, 1/500; Thermo Fisher Scientific), HA Tag Monoclonal Antibody (#26183, 1/10000; Thermo Fisher Scientific), anti-mouse IgG (#7076, 1/2000; Cell Signaling Technology), and anti-rabbit IgG (#7074, 1/2000; Cell Signaling Technology).

Immunofluorescence staining

The LX2 cells were treated with a washing step in TBST buffer, after which they were covered with 4% formaldehyde fixing solution and fixed at 4°C for 15 min. After the formaldehyde was removed, the cells were covered with sealing liquid and incubated for half an hour in a precisely regulated atmosphere of humidity and temperature (37°C) inside an incubator. The cells were then incubated with primary antibodies against YTHDF2 (ab246514, 1/50; Abcam) and α -SMA (ab7817, 1/1000; Abcam), followed by incubation with goat anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody (A-11029; Thermo Fisher Scientific) or goat anti-rabbit IgG (H + L) cross-adsorbed secondary antibody (A10520; Thermo Fisher Scientific). DAPI working solution (Beyotime) was added, and the mixture was incubated for 10 min at room temperature in the dark. After the DAPI was removed, anti-fluorescence attenuation tablets were added, and the images were viewed and acquired via a fluorescence microscope (Leica) [13].

m⁶A level detection

RNA from liver tissues and LX2 cells was isolated via TRIzol reagent (Beyotime). The binding solution (ab185912; Abcam) was added to each well, followed by the addition of RNA samples. After incubation for 90 min, the wells were washed with diluted 1× wash buffer, and diluted capture antibody was added. Then, the diluted detection antibody was added, and the mixture was incubated for 30 min. After that, the diluted enhancer solution was added, and the mixture was incubated at room temperature for 30 min. Then, 100 µL of developer solution was added to each well, and the mixture was incubated for 10 min in the dark. When the color of the positive control was turned medium blue, 100 µL of stop solution was added to stop the enzyme reaction. The absorbance was read at 450 nm with a microplate reader (Varioskan ALF; Thermo Fisher Scientific).

Methylated RNA immunoprecipitation

The RNA in the LX2 cells was extracted with TRIzol reagent. A/G Dynabeads were mixed with m⁶A antibodies before being added to the RNA samples. Elution buffer was added to the mixture, which was subsequently incubated for 5 min at 42°C. Soon afterward, further enrichment of the RNA was performed with the aid of the RNeasy MinElute Cleanup Kit (74204; Qiagen, Dusseldorf, Germany). The m⁶A level in ACSL4 mRNA was examined via qRT-PCR analysis.

RNA pull-down assay

ACSL4 was labeled with a biotin probe and then stirred with streptavidin magnetic beads (Thermo Fisher Scientific) at room temperature for 25 min. LX2 cells were lysed, mixed with the above mixture and incubated for 1 h. The complexes were then washed with 1× wash buffer, eluted with elution buffer, and stirred for 20 min at 37°C. The samples were added to reducing sample buffer, followed by heating for 5 min at 100°C. The expression of YTHDF2

in the sample complexes was measured via western blot analysis.

RNA immunoprecipitation (RIP) assay

LX2 cells were lysed in RNA immunoprecipitation (RIP) lysis buffer and then mixed with protein A/G beads coated with an anti-YTHDF2 antibody (or IgG, which served as the control). Then, a magnet was used to immobilize the magnetic bead-bound complexes and wash off the unbound materials. After the purification of RNA, the content of ACSL4 mRNA in the precipitation complexes was measured via qRT-PCR [14].

Polysome fractionation

LX2 cell suspension was prepared at a density of 1×10^5 cells/mL. The cells were mixed with cycloheximide (MKBio, Shanghai, China) and cultured for 15 min. The supernatant was removed after centrifugation. Next, the cell contents were lysed, and the supernatant of the cell lysate after centrifugation was poured off into another centrifuge tube. The obtained samples were placed on gradient sucrose and then placed in a SW41 rotor barrel and centrifuged at 222,227 g for 3 h at 4°C. Subsequently, the mRNA expression level of ACSL4 was detected via qRT-PCR after fractionation [15]: the nontranslating fraction (< 40S), translation-initiation fraction (including 40S ribosomes, 60S ribosomes, 80S monosomes, and < 80S), and translation-active polysomes (> 80S) were used.

Statistical analysis

GraphPad 8.0 was used to conduct the statistical analysis. For the comparisons between the two groups, when there was no significant difference according to the F test ($P \geq 0.05$), an unpaired *t*-test was performed. When there was a significant difference according to the F test ($P < 0.05$), an unpaired *t*-test with Welch's correction was conducted. For comparisons among three or more groups, one-way ANOVA followed by Tamhane's T2 multiple comparisons test or two-way ANOVA followed by Sidak's multiple comparisons test was performed. $P < 0.05$ was considered statistically significant.

Results

Downregulation of YTHDF2 alleviates HF

We first generated an HF mouse model. The staining results revealed an increase in fibrosis and inflammatory cell infiltration in liver tissues in the HF group (Figure 1A), which confirmed that the model was successfully established. Figure 1B shows that both the ROS and iron levels were increased in the HF model mice ($P < 0.001$), which preliminarily confirmed that the ferroptosis level was increased. In addition, the change in YTHDF2 was significantly upregulated after HF induction ($P < 0.001$; Figure 1C). YTHDF2 interference was subsequently performed in HF model mice (Figure 1D) to study the effect of YTHDF2 on HFs. The results revealed that fibrosis was weakened after YTHDF2 was downregulated (Figure 1E). Interference with YTHDF2 decreased the ROS and iron levels (Figure 1F). These findings indicate that YTHDF2 may be positively correlated with HFs in mice and may affect ferroptosis.

YTHDF2 promotes hepatic stellate cell activation *in vitro*

Given that TGF- β -induced hepatic stellate cell (HSC) activation plays an integral role in HF [16], we explored the potential role of YTHDF2 in the HSC line LX2 after TGF- β treatment. YTHDF2

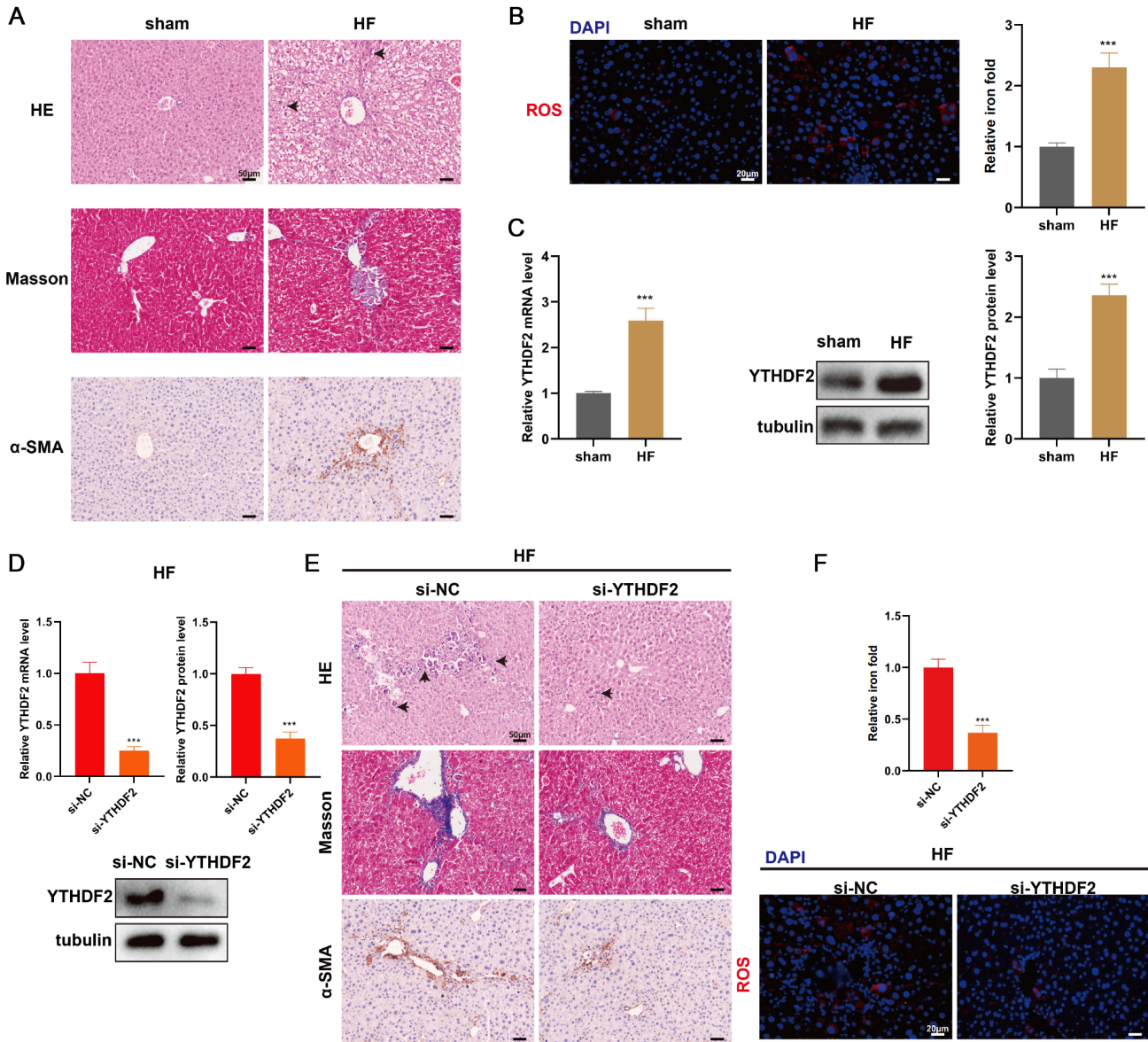


Figure 1. Effect of YTHDF2 on HF The liver tissues of C57BL/6 mice ($n = 12$) were collected. (A) HE staining (inflammation indicated by arrows), Masson staining (blue signal), and α -SMA immunohistochemical staining (brownish-yellow signal) were performed. Scale bar: 50 μ m. (B) ROS (blue signal: DAPI; red signal: ROS) and iron levels in liver tissues were detected. Scale bar: 20 μ m. (C) YTHDF2 levels in liver tissues were measured via qRT-PCR and western blot analysis. HF model mice were injected with YTHDF2-interfering adenovirus vectors (si-YTHDF2). (D) YTHDF2 levels in liver tissues were measured via qRT-PCR and western blot analysis. (E) HE staining (inflammation indicated by arrows), Masson staining (blue signal), and α -SMA immunohistochemical staining (brownish-yellow signal) were performed. Scale bar: 50 μ m. (F) ROS (blue signal: DAPI; red signal: ROS) and iron levels in liver tissues were detected. Scale bar: 20 μ m. *** $P < 0.001$. (B–D) Unpaired t -test with Welch's correction; (F) Unpaired t test.

regulates CBS expression in an m^6A -dependent manner in gastric cancer, indirectly altering the expression of ACSL4 [11], which is an important marker of ferroptosis. ACSL4 and α -SMA expression levels were downregulated after interference with YTHDF2 ($P < 0.05$; Figure 2A,B). In addition, YTHDF2 interference suppressed iron and ROS levels (Figure 2C). YTHDF2 was overexpressed, and ACSL4 was inhibited in LX2 cells (Figure 2D). The increases in α -SMA expression, iron content, and ROS levels induced by YTHDF2 overexpression were reversed by ACSL4 interference ($P < 0.01$; Figure 2E,F). These findings indicate the promotion effect of YTHDF2 on HSC activation.

YTHDF2 specifically binds to ACSL4 mRNA and regulates its expression

YTHDF2 is an important code-reading protein, and ACSL4 can be indirectly regulated by m^6A in gastric cancer. Hence, we hypothesized that the regulation of ACSL4 in HFs depends on YTHDF2-mediated m^6A modification. The m^6A levels in the mouse liver tissues and LX-2 cells were examined, and the results revealed that, in the HF model mice and the TGF- β -induced LX-2 cells, the m^6A levels were dramatically elevated (Figure 3A,B). RNA pull-down detection revealed that the YTHDF2 probe could bind to endogenous ACSL4 (Figure 3C). In addition, ACSL4 mRNA was enriched in

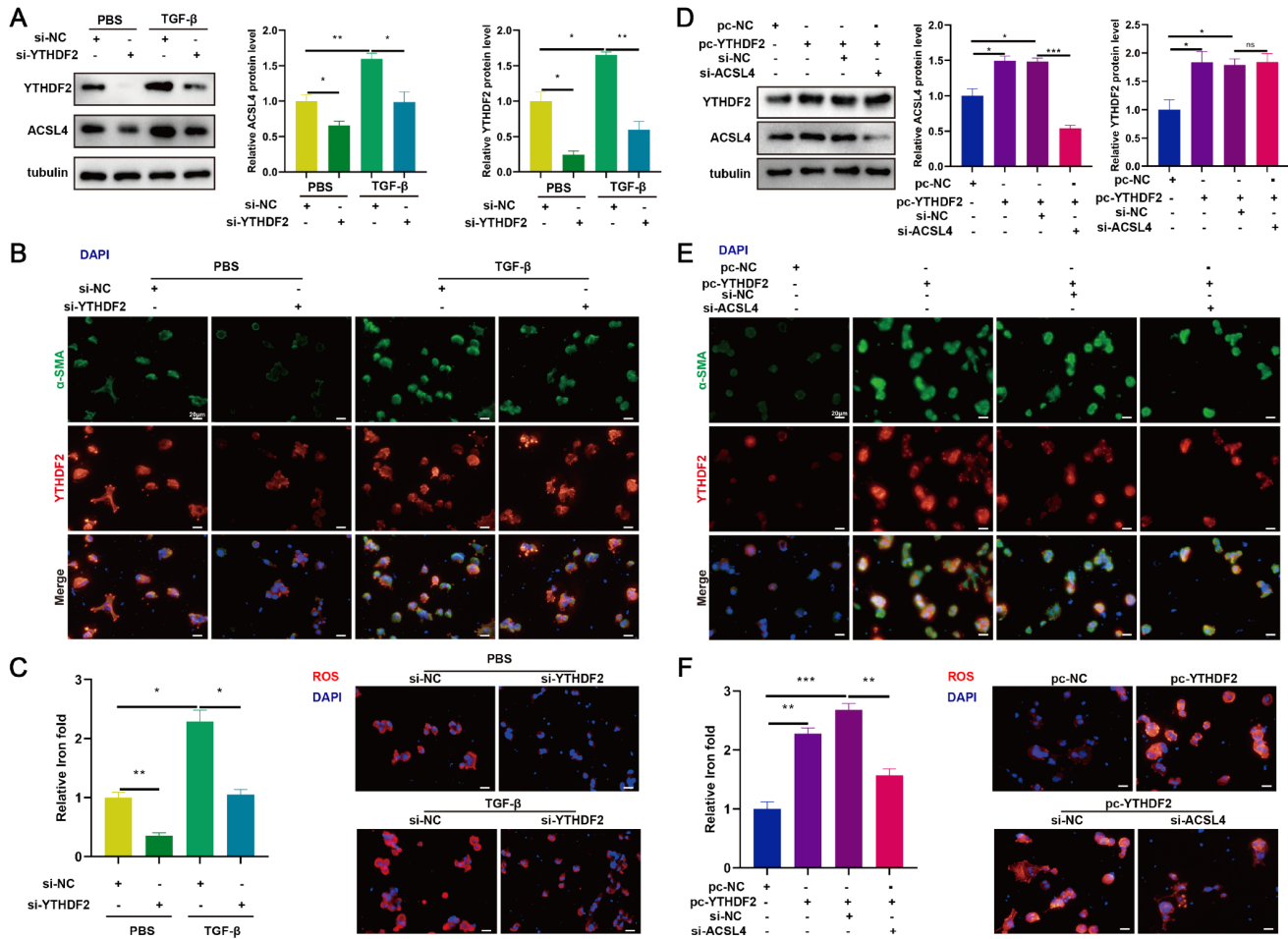


Figure 2. Effect of YTHDF2 on HSC activation LX2 cells were treated with TGF-β and then transfected with si-YTHDF2, si-ACSL4, or pc-YTHDF2, $n=3$. (A,D) The protein levels of YTHDF2 and ACSL4 in LX2 cells were measured via western blot analysis. Tubulin served as an internal control. (B,E) IF staining was performed to detect the expressions of YTHDF2 and α-SMA (blue signal: DAPI; red signal: YTHDF2; green signal: α-SMA). Scale bar: 20 μm. (C,F) Changes in ROS (blue signal: DAPI; red signal: ROS) and iron content were detected. Scale bar: 20 μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. One-way ANOVA followed by Tamhane's T2 multiple comparisons test was used.

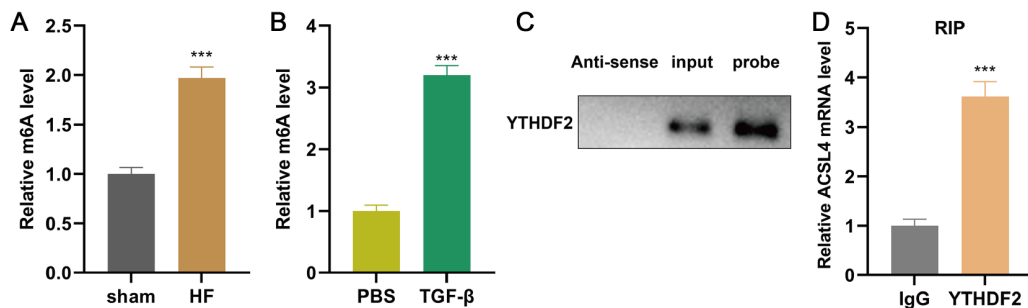


Figure 3. The binding relationship between YTHDF2 and ACSL4 An HF mouse model was generated, $n=6$. (A) Total m⁶A levels in the sham group and HF group were measured. LX2 cells were treated with TGF-β, $n=3$. (B) m⁶A levels in the PBS group and TGF-β group were measured. (C,D) RNA pull-down (C) and RIP (D) were performed to detect the interaction between YTHDF2 and ACSL4 mRNA. *** $P < 0.001$. (A,B,D) Unpaired t -test.

the immunoprecipitates containing YTHDF2 compared with the IgG immunoprecipitates ($P < 0.001$; Figure 3D). The above results confirm the regulation of ACSL4 expression by YTHDF2.

YTHDF2 modulates ACSL4 protein expression in an m⁶A-methylation-dependent manner

As shown in Figure 3, we verified that the expression of ACSL4 is

regulated by YTHDF2 and may be related to m⁶A modification. The m⁶A binding region mutant of YTHDF2, YTHDF2-WT/Mut, was constructed and transfected into LX2 cells. Western blot analysis of the changes in YTHDF2 and ACSL4 expressions revealed that YTHDF2-Mut did not affect the expression of YTHDF2 ($P > 0.05$; Figure 4A) but reduced the expression of ACSL4 ($P < 0.001$; Figure 4A). The RIP results indicated that the interaction between YTHDF2

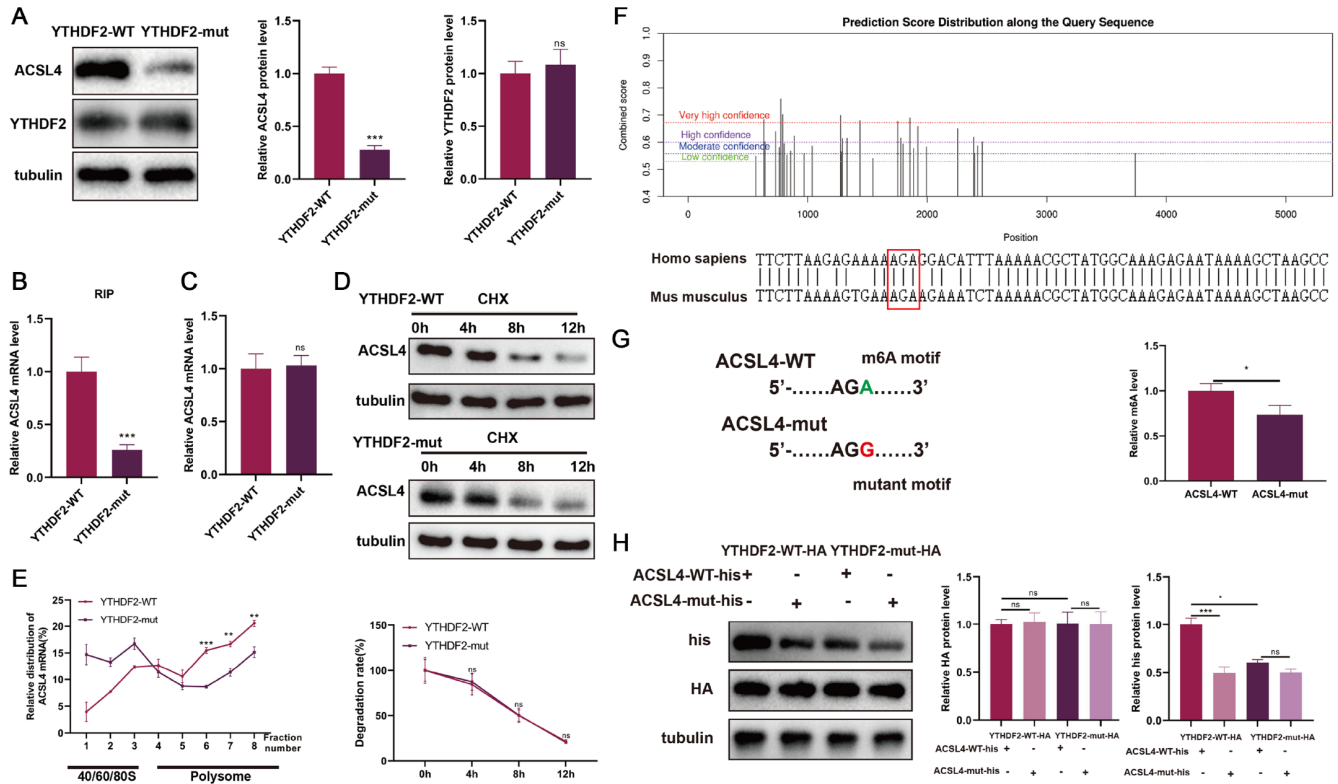


Figure 4. Mechanism by which YTHDF2 regulates ACSL4 The m⁶A binding region of YTHDF2, YTHDF2-WT/mut, was constructed and transfected into LX2 cells, $n=3$. (A) The protein levels of YTHDF2 and ACSL4 were detected via western blot analysis. Tubulin served as an internal control. (B) RIP analysis was performed to detect the interaction between YTHDF2 and ACSL4 mRNA after transfection. IP: YTHDF2. (C) ACSL4 mRNA levels were detected via qRT-PCR. LX2 cells were treated with 0.1 mg/ml CHX to block translation. (D) ACSL4 protein levels were detected by western blot analysis. Tubulin served as an internal control. The degradation rate (%) of the ACSL4 protein was measured. (E) Polysome fractionation-qPCR was performed to detect the translation of ACSL4. (F) Schematic diagram of the m⁶A modification site in the ACSL4 mRNA sequence. The m⁶A motif at 204 bp in the 5'UTR of ACSL4 was mutated. (G) MeRIP assay was performed to detect m⁶A level. (H) The protein levels of his (ACSL4) and HA (YTHDF2) were detected by western blot analysis. Tubulin served as an internal control. ns, not significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (A–C,G) Unpaired t -test; (D,E) two-way ANOVA followed by Sidak's multiple comparisons test; (H) one-way ANOVA followed by Tamhane's T2 multiple comparisons test.

and ACSL4 was weakened after infection ($P < 0.001$; Figure 4B). Moreover, the mRNA expression of ACSL4 did not significantly change ($P > 0.05$; Figure 4C). We hypothesized that YTHDF2 positively regulates ACSL4 expression by promoting its protein expression and that this regulation involves m⁶A methylation, which may take place during translation into proteins. To determine whether this mechanism functions through promoting protein production or inhibiting protein degradation, we treated hypoxic LX2 cells with 0.1 mg/mL CHX (cycloheximide, C4859; Sigma-Aldrich) and found that the protein content of the mutant YTHDF2 was unchanged after the translation was blocked ($P > 0.05$; Figure 4D). Therefore, we believe that this mechanism is not achieved primarily through the YTHDF2-mediated inhibition of protein degradation. Polysome fractionation revealed that ACSL4 mRNA was more enriched after 80 s ($P < 0.01$; Figure 4E), which proved that YTHDF2 could promote translation. Figure 4F shows a graphic representation of the m⁶A modification site located within the ACSL4 mRNA sequence. It has been reported that m⁶A methylation can enhance translation via the 5'UTR. Combined with our prediction results, the m⁶A motif located in the 204 bp 5'UTR was selected for mutation (AGA→AGG). Methylated RNA immunoprecipitation (MeRIP) experiments revealed that m⁶A methylation at the Mut site of ACSL4 was significantly reduced ($P < 0.05$; Figure

4G), indicating that the mutation location affected m⁶A. Moreover, the ACSL4-WT (his) and ACSL4-Mut (his)/YTHDF2 (HA) vectors expressed the same amount of ACSL4 without external influence, and ACSL4 was expressed at the same level as YTHDF2-Mut (Figure 4H). The above data indicate that YTHDF2 promotes the translation of ACSL4 mRNA via m⁶A.

Overexpression of ACSL4 reverses the effect of YTHDF2 knockdown on HFs in mice

We then investigated the effect of ACSL4 overexpression on HF model mice. AAV9-si-YTHDF2 and AAV9-oe-ACSL4 were injected into HF mice (Figure 5A). Figure 5B shows that interference with YTHDF2 reduced iron and ROS levels, whereas ACSL4 overexpression reversed these effects ($P < 0.001$). Tissue immunostaining revealed that fibrosis and inflammatory infiltration were decreased after YTHDF2 interference, whereas these effects were weakened after ACSL4 overexpression (Figure 5C). These results indicate that ACSL4 overexpression reverses the attenuation of HFs after YTHDF2 knockdown.

Discussion

Chronic hepatic disease is a cause of HF that occurs during liver injury repair and healing. There is still no good treatment for HF

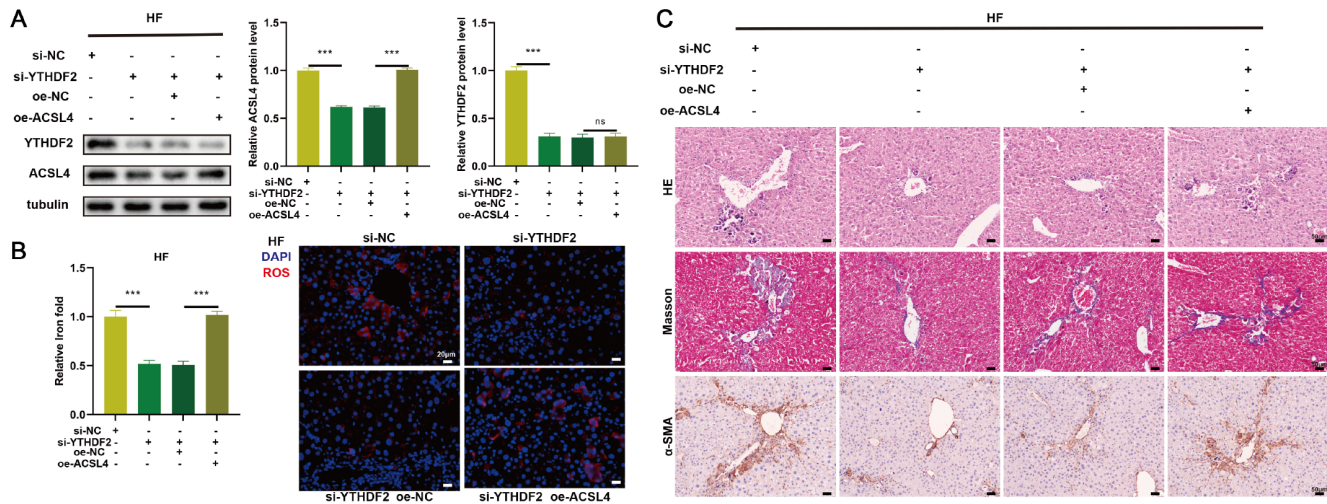


Figure 5. Effect of ACSL4 on HF in mice AAV9-si-YTHDF2/NC and AAV9-oe-ACSL4/NC were injected into HF mice through the tail vein, $n = 12$. (A) The protein levels of YTHDF2 and ACSL4 in the mouse liver were detected by western blot analysis. Tubulin served as an internal control. (B) ROS (blue signal: DAPI; red signal: ROS) and iron levels in mouse livers were measured. Scale bar: 20 μm . (C) HE staining, Masson staining (blue signal), and α -SMA immunohistochemical staining (brownish-yellow signal) were performed. Scale bar: 50 μm . ns, not significant. *** $P < 0.001$. (B) One-way ANOVA followed by Tamhane's T2 multiple comparisons test.

available to date [17]. In this work, we constructed an HF mouse model via the intraperitoneal injection of CCl_4 , and the cell model was induced with TGF- β . The increased inflammatory infiltration and HF suggest successful modeling. Furthermore, YTHDF2 expression was found to be dramatically upregulated in the HF models, and the inhibition of YTHDF2 attenuated HF, as well as ferroptosis. In addition, we verified the interaction between YTHDF2 and ACSL4, which was mediated by m^6A .

m^6A has been linked to various cell activities and plays an important role in influencing the progression of hepatic disorders; for example, high m^6A methylation level may alleviate lipopolysaccharide-induced hepatic injury [18]. Research has shown that differential m^6A methylation is involved mainly in the immune response and apoptosis-related links during the reversal of HF [19]. The m^6A binding protein YTHDF3 was reported to directly regulate PRDX3 translation in an m^6A -dependent manner, thus affecting its function in HF [10]. In addition, the m^6A binding protein YTHDF2 is directly regulated by miR-145, which further enhances HCC proliferation [20]. In this study, we found that YTHDF2 was highly expressed in CCl_4 -induced model mice and that the knockdown of YTHDF2 reduced the expressions of genes associated with HSC activation and fibrosis. In TGF- β -induced LX2 cells, interference with YTHDF2 reduced HSC activation, whereas overexpression of YTHDF2 promoted it.

Ferroptosis is an iron-dependent form of programmed cell death mediated by the accumulation of lipid peroxides and is genetically and biochemically distinct from other forms of regulatory cell death [2]. Ferroptosis plays an important regulatory role in the occurrence and development of many cancers, acute kidney injury, inflammation, hemolytic diseases, nervous system diseases, ischemia/reperfusion, and other diseases. Activating or blocking the ferroptosis pathway can slow the progression of a disease, providing a promising strategy for the treatment of many diseases [21]. High levels of iron in the liver and ferroptosis can promote HF in mice [22,23]. In our research, the iron and ROS levels were elevated in both CCl_4 -induced mouse liver tissues and TGF- β -induced LX2 cells, whereas YTHDF2 inhibition reduced their levels. These data suggest

that interference with YTHDF2 decreases ferroptosis.

ACSL4 is an enzyme that freely switches long-chain fatty acids to fatty acyl-CoA esters, thus participating crucially in the metabolism of lipids and fatty acids for their breakdown. The protein ACSL4 is crucial for ferroptosis [24]. The inhibition of ACSL4 helps reduce renal ferroptosis and inflammation and mitigate acute kidney injury [25], whereas activation of ACSL4 contributes to the occurrence of ferroptosis [26]. ACSL4 also acts as a key factor involved in ferroptosis in ischemic stroke [27]. Moreover, in HCC, a reduction in ACSL4 inhibited ferroptosis, as well as fibrosis [6]. Similar to the findings of a previous study, we discovered that the inhibition of ACSL4 suppressed HSC activation and ferroptosis. Furthermore, we discovered that YTHDF2 specifically binds to ACSL4 mRNA and regulates its expression and that this regulation is achieved through the m^6A mechanism. *In vivo* experiments revealed that the overexpression of ACSL4 reduces the attenuating effect of the downregulation of YTHDF2 on fibrosis.

In summary, our work is the first to show that YTHDF2 affects HF. In addition, we found for the first time that YTHDF2 mediates ACSL4 expression through an m^6A -dependent mechanism. We also found that YTHDF2 may mediate the expression of ACSL4 in an m^6A -dependent manner to influence ferroptosis, and thus regulate HF. Our study may provide a new theoretical basis for the treatment of HF.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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