miR-186-5p Prevents Hepatocellular Carcinoma Progression Through Targeting METTL3 to Regulate m6A-Mediated Stabilization of FSTL5

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Primary research

Keywords: microRNA, hepatocellular carcinoma, METTL3, FSTL5, mRNA stability

Posted Date: June 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-598803/v1>

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Abstract

Background: Hepatocellular carcinoma (HCC) processes in multi-steps which involves the sophisticated interactions of genetics, epigenetics, and transcriptional changes. According to before investigations, methyltransferase-like 3 (METTL3)-mediated m6A modification regulates the development of various cancers by regulating gene stability. However, the studies focusing on miRNA's regulatory effect of N6-methyladenosine (m6A) modification on HCC progression are still limited.

Methods: Immunohistochemistry (IHC) staining detected the histopathological changes in the tumor tissues. Cell Counting Kit-8 (CCK-8), clone formation, and transwell assay investigated the changes in cancer cell proliferation, invasion, and migration. The RNA m6A level was confirmed by methylated RNA immunoprecipitation. The RNA stability assay indicated the half-life ($t_{1/2}$) of RNA in HCC cells. The prognosis of the indicated patients' cohort was analyzed using the cancer genome atlas (TCGA) datasets. Luciferase report analysis was used to study the potential binding between microRNA (miRNA) and mRNA. A mice tumor transplant model was further established to study the changes in tumor progression.

Results: Follistatin-like 5 (FSTL5) was found to be significantly downregulated in HCC, and it inhibited the further progression of HCC. The RNA stability analysis indicated that the mRNA $t_{1/2}$ gene of HCC cells was shortened. Besides, METTL3 reduced the stability of FSTL5 mRNA in a m6A-YTH domain family 2(YTHDF2)-dependent manner. Functional experiments revealed that the downregulated METTL3 inhibited the HCC progression by up-regulating FSTL5 *in vitro* and *in vivo*. Luciferase report analysis confirmed that miR-186-5p directly targeted the METTL3. Additionally, miR-186-5p inhibited the proliferation, migration, and invasion of HCC cells by downregulating METTL3. We identified that miR-186-5p prevented the HCC progression by targeting METTL3 to regulate m6A-mediated FSTL5 stabilization.

Conclusions: The miR-186-5p/METTL3/YTHDF2/FSTL5 axis perhaps point out a new direction for the targeted therapy of HCC.

Introduction

Hepatocellular carcinoma (HCC), a deadly cancer, ranks sixth in the common types of cancer globally [1, 2]. HCC might be induced by nonalcoholic fatty liver disease, type 2 diabetes mellitus, obesity and etc., making the incidence of HCC grow annually [3]. The clinical treatment of HCC is limited because of its inconspicuous early symptoms and late diagnosis [4]. Thus, it is of vital importance to understand the molecular mechanism of HCC pathogenesis to provide a direction for HCC diagnosis and therapy.

The follistatin family is a kind of extracellular matrix glycoprotein. Its participation in physiological processes, inflammatory response, tissue remodeling, and embryonic development has been verified [5]. As a member of the follistatin family, FSTL5 is a protein that directly binds to activin [6]. More and more studies have shown that FSTL5 also participated in regulating tumor occurrence. Remke M et al. found

that FSTL5 was a poor prognostic marker of non-WNT/non-SHH medulloblastoma [7]. FSTL5 may become an attractive therapeutic target and prognostic indicator [8]. However, the potential mechanism of FSTL5 in HCC still needs further exploration.

More studies have shown that gene mutations and epigenetic regulations, including methylation, chromatin modification, and remodeling, are the drivers of HCC [9]. N6-methyladenosine (m6A) RNA methylation, a most abundant RNA modifications, plays a critical role in tumor progression [10, 11]. m6A modification regulates gene expressions by regulating the mRNA stability, splicing, translation, and microRNA maturation [12]. m6A modification is mediated by m6A “writers”, “erasers”, and “readers”, which function in adding, removing, and recognizing m6A modifications, respectively [13]. METTL3, the main component of m6A “writers”, promotes m6A RNA modification, which is recognized by m6A “readers”, including the YTHDF, heterogeneous nuclear ribonucleoprotein A2B1 (hnRNP A2B1), and insulin-like growth factor 2 mRNA-binding protein 2 (IGF2BP) [14, 15]. It has been found that m6A “writers”, “erasers”, and “readers” function vital in regulatory in the pathogenesis of cancers, including bladder cancer, gastric cancer, colorectal cancer, and HCC [16–19].

MicroRNA (miRNA) is an endogenous small non-coding RNA with 19–22 nucleotides; it can down-regulate gene expression after transcription via targeting the 3'UTR gene [20]. miRNAs participate in various cancer development and progression [21]. Recently, miRNAs were found to be regulated by METTL3 through m6A modification to influence the tumor genesis and development [22]. However, the studies on whether miRNAs could regulate the m6A process in HCC development are still limited. Here, we identified that FSTL5 was downregulated in HCC and reported that FSTL5 has anti-tumor effects on HCC. Besides, METTL3 promoted the FSTL5 mRNA degradation, and miR-186-5p negatively regulated the expression of METTL3. More importantly, miR-186-5p, as concluded, inhibited the progression of HCC by downregulating METTL3, leading to FSTL5 upregulation by decreasing the m6A modification of FSTL5.

Materials And Methods

Tissue sample collection

Twenty-eight pairs of HCC tissues and adjacent non-tumor tissues were acquired from the First Affiliated Hospital of Bengbu Medical College from January 2017 to January 2018. The agreement was signed with the approval of its Ethics Committee (No. 2017059). All patients involved in this study did not receive chemotherapy or radiotherapy before the operation. The **Supplementary Table 1** shows the characteristics of all participants.

Cell culture and transfection

The human HCC cell lines HepG2 and Huh-7 were purchased from American Type Culture Collection (ATCC, USA) and cell bank of Chinese Academy of Sciences (Guangzhou, China), respectively. Both cells were grown in a Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37°C in a water-saturated atmosphere with 5% CO₂. For in

vitro transfection, Lipofectamine 2000 (Invitrogen, USA) was used to help transfer 50nm mimic control, miR-186-5p mimic, or inhibitor (Ribo bio, Shanghai, China) into the cells. For the overexpression of FSTL5, METTL3, or YTHDF2 in cells, the cDNA of human FSTL5, METTL3, and YTHDF2 was cloned into pLV-EGFP(2A) Puro vector by Genepharma (Guangzhou, China), respectively. The short hairpin RNA (shRNA) against METTL3 and FSTL5 was from Genepharma (Guangzhou, China). The **Supplementary Table 2** shows the sequences for transfection.

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

The total RNA was separated by a Trizol reagent (Invitrogen, USA). The concentration of RNA was detected using NanoDrop (Thermo, USA). We quantified the miRNAs and mRNAs using TaqMan miRNA probes (Applied Biosystems, USA) and Synergy Brands Synergy Brands (SYBR) Green Dyes (Ambion, USA), respectively. We used the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme, China) and the main champ SYBR qPCR mixture (Vazyme, China) for retroviral polymerase chain reaction and quantitative polymerase chain reaction, respectively. The calculated equation is shown below: $\Delta\Delta CT = (CT_{\text{miRNA or mRNA}} - CT_{\text{U6 or GAPDH}})_{\text{tumor}} - (CT_{\text{miRNA or mRNA}} - CT_{\text{U6 or GAPDH}})_{\text{control}}$. The primers are shown in **Supplemental Table 3**.

Western blot

Radio-Immunoprecipitation Assay (RIPA) lysis buffer and PMSF (Beyotime, China) extracted the total protein in the tissue or cell of tumor. The total protein concentration was determined using a BCA detection kit (Santa Cruz, USA). After electrophoresis, the prepared protein sample was transferred to PVDF, where it hatches along with the prepared antibody. Finally, an enhanced chemiluminescence (ECL, ThermoFisher, USA) was used to visualize the membrane. ImageJ software was used to analyze the protein bands. The antibodies against METTL3 and GAPDH were from CST (Beverly, USA). The antibodies against FSTL5, methyltransferase-like 14 (METTL14), AlkB homolog 5 (ALKBH5), and Fat mass and obesity-associated protein (FTO) were purchased from Abcam (Cambridge, USA).

CCK-8 assay

The cells were placed in a 96-well plate, with 1×10^4 cells/well. Then, 10 μL of CCK-8 solution was added to the cell for 1 h at 37°C . The light density of the cells was measured at 450 nm by a microchannel plate reader.

Clone formation assay

The cells were digested with 0.25% trypsin, plated in a 6-cm culture dish at a density of 2×10^3 /well, and incubated at 5% CO_2 and 37°C for two weeks. Next, the cells in the dish were washed with PBS, fixed with 5 mL of methanol for 15 min, and subjected to Giemsa dyeing for another 15 min. The colonies was observed and counted using an optical microscope.

Cell migration and invasion assay

Transwell assay measured cell migration and invasion. After diluting (1:4) BD glue (BD biosciences, USA) with Opti-MEM™ I (Gibco, USA), add 50ul Matrigel into the upper chamber. HepG2 and Huh-7 cells were harvested and inoculated in an 8-mm well transmembrane plate in the upper transmembrane chamber at a density of 50,000 cells/well (Costar, USA) pre-treated with Matrigel or not. The upper and lower chambers each were added serum-free medium and 20% FBS medium. After 24 h incubation, the unmigrated or uninvaded cells were wiped out cotton swabs. Pictures were taken, and the number of cells was calculated. The average value of cells number was calculated after counting the photos of 6 fields.

Luciferase reporter assay

The wild-type or mutant 3'UTR of METTL3 was amplified and cloned separately into pGL3-basic vector (Promega, USA). The binding site of the 3'UTR of METTL3 was mutated from UGUACAAUAGCUUUCUUCUUUAU to UGUACAAUAGCUUCAAGAAAAU for miR-186-5p. Then, the liver cancer cells were placed in a 24-well plate and dyed with a wild or mutant luciferase plasmid and miR-186-5p mimic, miR-186-5p inhibitor. A Dual-Luciferase Reporter Assay System (Promega, USA) measured the activity of the two fluorescent enzymes.

m6A RNA methylation quantification

An m6A RNA methylation analysis kit (Abcam, USA) was used to measure the total methylation content of m6A. Briefly, after adding the detection antibody solution in each reaction, 200 ng of the total RNA was added in each reaction. The m6A level was quantified by coloration, and the absorbance of each reaction was measured at 450 nm. For the measurement of FSTL5 m6A enrichment, the total RNA of HepG2 cells was extracted; then, immunoprecipitation was done using a Magna MeRIP m6A kit (Merck millipore company, Germany) with protein A beads combined with m6A antibody in advance. The partial RNA was analyzed by RT-qPCR after immunoprecipitation.

RNA immunoprecipitation-RT-PCR

The HepG2 cells were transfected with YTHDF2 overexpression lentivirus. The virus was washed twice with PBS, and it was dissolved in a lysine buffer with a protease inhibitor mixture and ribozyme inhibitor for 30 min at 4°C. The cytolitic enzyme was centrifuged, and the filtrate was collected. The supernatant solution was heated for 4 h with magnetic beads (Sigma, USA), which were pre-processed by IgG or YTHDF2 antibodies (Abcam, USA); then, it was washed five times with a wash buffer (50 mM Tris, 0.5 mM DTT, RNase inhibitor, 200 mM sodium chloride, 0.05% NP40, 2 mM ethylamine tetraethyl). Then, the beads were washed in a solution containing 0.1% sodium dodecyl sulfate and 10 mL of potassium enzyme and hatched for 30 min at 55°C. The imported nucleic acids and immune precipitates were separated using Trizol reagents (Invitrogen, USA) and analyzed quantitatively by a chain reaction of polymerase and gel electrophoresis of agarose.

RNA Stability

The transfection of HepG2 cells with sh-NC was done to detect the stability of RNA in cells. sh-METTL3, the control lentivirus, or the YTHDF2 overexpression lentivirus for 24 h; then, 5 mg/mL actinomycin D (Sigma, USA) treated the cells. RNA isolation was done with a Trizol reagent (Invitrogen, USA) at 0, 3, and 6 h. Then, it was normalized to GAPDH by real-time polymerase chain reaction analysis. The $t_{1/2}$ of mRNA was calculated.

Immunohistochemical (IHC) analysis

The tumor tissues covered with paraffin were waxed and treated with 3% H₂O to disable the endogenous peroxidase. After 1 h of blocking the bond with non-specific antigens at 37°C with 5% cow serum albumin, the specific primary antibodies (1:100 diluted, Abcam, USA) against FSTL5, METTL3, or YTHDF2 were incubated at 4°C for 12 h. About 1 h after hatching the second antibody at 37°C, the slice was dyed with diamino-ventylamine and dyed again in a small-purpose. A representative image was taken with an optical microscope (Olympus, Japan).

Tumor xenograft model

About 1×10^7 HepG2 cells were suspended in 200 μ L of PBS and injected into each side of BALB/c nu/nu male mice in the age of 4-6 weeks (Charles River Lab, Beijing, China). The tumor volume in mice was recorded every three days (volume = (length $\times$ width ²)/2). The knockdown of METTL3 and FSTL5 was performed using METTL3 and FSTL5 knockdown lentivirus (GenePharma, China), respectively, by intratumoral injection of 50 μ L of virus (4×10^7 IU/mL) after tumor cell injection. After 15 days, the mice were killed. Western blot analysis was carried out on the tumor tissues. Euthanasia method of mice: put the mice into the box, inject carbon dioxide into the box at the rate of 20% per minute, observe that the mice have no breathing, turn off carbon dioxide, and check again to confirm that the mice have died (no heartbeat, pupil dilation). This animal experiment was consented by the Animal Health and Use Committee of the First Affiliated Hospital of Bengbu Medical College.

Hematoxylin and eosin (HE) staining

The tumor tissues were fixed in 4% paraformaldehyde (Sigma, USA) for 48 h and embedded in paraffin. Thereafter, the tissues were sliced, and the sections were dyed with hematoxylin for 2 min and eosin for 1 min. The images were viewed using a light microscope (Nikon, Japan).

Statistical analysis

The Prism Graphpad 8.0 software analyzed the data, which are in the form of mean values. Two-tailed Student's t-test compared the two groups. One-way ANOVA compared multiple groups. $p < 0.05$ showed statistical significance.

Results

FSTL5 inhibits HCC proliferation, migration, and invasion

The FSTL5 expression level was identified in tissues and cell lines of HCC for finding out its effects. And we found that its expression in adjacent non-tumor tissues was vitally higher than that in HCC tissues (Fig. 1A, B). Similarly, the level of FSTL5 in HCC cell line Hh-7 was downregulated in MHCC97-H, HepG2, SMMC-7721 cells (Fig. 1C, D). Since the average expression of FSTL5 was higher in Huh-7 and HepG2 cells than in other HCC cells, these two cell lines were chosen for subsequent experiments. Then, the FSTL5 overexpression plasmid was transfected into Huh-7 and HepG2 cells. RT-qPCR analysis confirmed the transfection efficiency of FSTL5 (Fig. 1E). The results indicated that the overexpression of FSTL5 inhibited the cell viability, clone formation, migration, and invasion in Hh-7 and HepG2 cells (Fig. 1F-H).

Mettl3 Promotes Fstl5 Mrna Degradation

Then, we investigated why FSTL5 was downregulated in HCC and served as a suppressor. The RNA stability test showed that the $t_{1/2}$ gene of HCC cells was shortened, suggesting the possible presence of RNA modification (Fig. 2A). m6A is a relatively common chemical formula in eukaryotes and plays an important role in many biological processes. Besides, m6A modification regulates the mRNA stability to regulate the gene expression in cancer [23, 24]. Then, we detected the m6A level in HCC tissues. We found that the total m6A level in adjacent non-tumor tissues was lower than that in tumor (Fig. 2B). The m6A modification was mediated by m6A methyltransferases METTL3 and METTL14, which were cleared by fat mass and FTO [25, 26]. Interestingly, only METTL3 expression was markedly increased in HCC (Fig. 2C). We assumed that the upregulated METTL3 might regulate the stability of FSTL5 by m6A modification in HCC. MeRIP qPCR analysis was used to assess the impact of METTL3 on m6A modifications aimed at the FSTL5 mRNA. As a result, m6A-specific antibodies were significantly enriched with the FSTL5 gene compared to the immunoglobulin group. Besides, the inhibition of METTL3 significantly reduced the m6A level of FSTL5 mRNA (Fig. 2D). Additionally, the knockdown of METTL3 extended the mRNA $t_{1/2}$ (Fig. 2E). Then, we found that METTL3 negatively regulated the FSTL5 expression of HCC cells, as indicated by RT-qPCR and western blot assays (Fig. 2F, G). We constructed a luciferase reporter plasmid containing the wild type and 3'UTR mutant of FSTL5. Luciferase reporter gene analysis showed that METTL3 inhibition significantly increased the activity of wild-type luciferase but not that of the mutant FSTL5 3'UTR, indicating that the adjustment of FSTL5 representation was affected by the m6A trim associated with METTL3 (Fig. 2H). Moreover, RT-qPCR and IHC assays indicated that METTL3 expression was increased in HCC tissues (Fig. 2I, J). Pearson's correlation assay indicated a negative correlation between METTL3 and FSTL5 expression in HCC (Fig. 2K). In summary, our findings show that the METTL3-mediated m6A formula inhibits the expression of FSTL5.

YTHDF2 promotes the FSTL5 mRNA degradation in a m6A-dependent manner

Recent studies revealed that YTHDF1/2/3, a unique m6A reader family, can target thousands of genes by identifying m6A sequencing [27]. Therefore, it is unsure whether YTHDF2 participated in the FSTL5 transcription expression of HCC cells inhibited by METTL3. Western blot showed that YTHDF2 overexpression decreased the FSTL5 protein expression (Fig. 3A). RIP-RT-PCR illustrated that YTHDF2 directly interacted with the FSTL5 mRNA; this shows that YTHDF2 can target the FSTL5 gene (Fig. 3B). The RNA stability assay indicated that the mRNA t_{1/2} was shortened by YTHDF2 overexpression (Fig. 3C). We further found that only the full length or wild type of the CDS region of FSTL5 mRNA could pull down the YTHDF2 protein, which was detected by immunoblot, indicating that YTHDF2 regulated the stability of FSTL5 mRNA by binding to the CDS region of FSTL5 (Fig. 3D). Moreover, YTHDF2 was found upregulating in HCC tissues (Fig. 3E). TCGA dataset analysis revealed that the high expression of METTL3 and YTHDF2 indicated the poor prognosis of HCC (Fig. 3F). Overall, METTL3 inhibited the expression of FSTL5 by YTHDF2-dependent FSTL5 mRNA degradation.

Downregulated METTL3 inhibits HCC cell proliferation, migration, and invasion by up-regulating FSTL5

To further assess whether METTL3 regulated the FSTL5 in HCC, METTL3 and FSTL5 were stably knocked down in Huh-7 and HepG2 cells. We used two sequence-specific shRNAs to exclude the off-target effect. The transfection efficiency of sh-METTL3-1, sh-METTL3-2, sh-FSTL5-1, and sh-FSTL5-2 in Huh-7 and HepG2 cells was confirmed by RT-qPCR assay (Fig. 4A and S1A). Functionally, METTL3 inhibition was found to significantly inhibit the proliferation of Huh-7 and HepG2 cells. However, the co-transfection of sh-FSTL5 reversed the reduced cell proliferation (Fig. 4B-C and S1B-C). Consistently, sh-METTL3 had a significant inhibitory effect on cell migration and invasion, which was fully demonstrated by transwell assay, while sh-FSTL5 antagonized the impact of sh-METTL3 (Fig. 4D and S1D). Namely, METTL3 served as a facilitator in HCC by negatively targeting FSTL5.

Downregulated METTL3 inhibits HCC tumor growth by up-regulating FSTL5 in vivo

To further confirm the role of METTL3/FSTL5 on HCC tumor growth, an *in vivo* xenotransplantation model was realized. The HepG2 cells were subcutaneously inoculated in nude mice. Based on the results, we can see that knocking out METTL3 has an obvious inhibitory effect on tumor growth and xenograft size, while co-transfection with FSTL5 silencing promoted the tumorigenicity (Fig. 5A and B). Furthermore, sh-METTL3 led to the augment of FSTL5, while FSTL5 silencing reversed the upregulated expression caused by sh-METTL3 (Fig. 5C). Besides, HE and IHC staining showed that the knockdown of METTL3 inhibited the tumor infiltration and expression of proliferative protein Ki67, while FSTL5 silencing reversed the role of sh-METTL3 (Fig. 5D). Above all, these results suggested that the downregulated METTL3 inhibited the HCC tumor growth by up-regulating FSTL5 *in vivo*.

A tumor xenograft model was constructed through the subcutaneous injection of 1×10^7 HepG2 cells. The knockdown of METTL3 and FSTL5 was performed using the METTL3 knockdown lentivirus and FSTL5 knockdown lentivirus (GenePharma, Shanghai, China), respectively, by the intratumoral injection of 50 μ L of virus (4×10^7 IU/mL) after the tumor cell injection. (A) Pictures of the isolated tumors of the

indicated group. $n = 5$. (B) The tumor of mice in the indicated group is shown. $n = 5$. (C) The expression of METTL3, FSTL5, and GAPDH in the tumor tissues were detected by western blot assay. (D) HE and IHC staining were used to assess tumor infiltration and Ki67 expression. Bar = 200 μm . The data represent three separate experiments and are expressed as mean $\pm$ SEM. *** $p < 0.001$, * $p < 0.05$, ** $p < 0.01$.

Mir-186-5p Inhibits Hcc Cell Proliferation, Migration, And Invasion

The protein and mRNA expression of METTL3 was increased in HCC tissues. There was no link between the METTL3 protein and gene expression in the raise rate of patients in each group (Fig. 6A). The mismatch between the METTL3 protein and mRNA expression levels in HCC suggests that METTL3 is involved in the development of HCC through post-transcriptional translation. As an important member of post-transcriptional regulatory factors, miRNA plays an indispensable role in HCC [28]. To further assess the molecular mechanism of METTL3 that participated in the occurrence of HCC, online databases Targetscan, miRWalk, and Starbase were used to predict the possible miRNAs (Fig. 6B). We found that miR-186-5p was the common miRNA in the three databases (Fig. 6B). Besides, the RT-qPCR analysis showed that miR-186-5p indicates the down control in HCC tissues. This shows that miR-186-5p downregulation can act on the mechanism of the onset of HCC disease (Fig. 6C). This data inspired us to study the role of miR-186-5p in HCC. We used the miR-186-5p mimic and inhibitors to raise or inhibit the miR-186-5p of cells, respectively (Fig. 6D). The clone formation assay showed that the overexpression of miR-186-5p inhibited the clone formation in Huh-7 and HepG2 cells, while the inhibition of miR-186-5p increased the clone formation in Huh-7 and HepG2 cells (Fig. 6E). Transwell assay showed that the overexpression of miR-186-5p inhibited Huh-7 and HepG2 cell migration and invasion, while the inhibition of miR-186-5p promoted cell migration and invasion (Fig. 6F).

Mettl3 Is A Direct Target Of Mir-186-5p

In HCC cells, miR-186-5p decreased and inhibited the m6A level (Fig. 7A). As shown in the western blot assay, miR-186-5p negatively regulated the METTL3 expression in HCC cells (Fig. 7B). We also provided the potential binding sites between the miR-186-5p and 3'UTR of METTL3 (Fig. 7C) and constructed luciferase reporter plasmids containing the wild type and synaptic variants of the 3'UTR of METTL3. The fluorescent enzyme analysis shows that miR-186-5p significantly reduced the fluorescent enzyme activity of wild METTL3 3'UTR rather than that of the mutant, indicating that the specificity of miR-186-5p combines the 3'UTR of METTL3 (Fig. 7D). Western blot further showed that the miR-186-5p mimic downregulated the METTL3 expression and upregulated the expression of FSTL5, which were reversed by the overexpression of METTL3 (Fig. 7E). The MeRIP qPCR assay also revealed that the miR-186-5p mimic significantly reduced the m6A level of FSTL5 mRNA, while that co-transfected with METTL3 led to an obvious increase (Fig. 7F). Therefore, through our research, we can see that miR-186-5p affects the modification of FSTL5 mRNA gene mediated by METTL3.

MiR-186-5p inhibits HCC cell proliferation, migration, and invasion by downregulating METTL3

We then verified whether miR-186-5p regulated the HCC cell malignancy by regulating METTL3. CCK-8 and clone formation assay showed that miR-186-5p inhibited the HCC cell visibility and clone formation, while the overexpression of METTL3 reversed the role of miR-186-5p (Fig. 8A, B). Transwell assay showed that the overexpression of METTL3-inhibited miR-186-5p inhibited the migration and invasion of HCC cells (Fig. 8C). It can be seen from these data that the malignant degree of HCC cells is regulated by miR-186-5p through the expression of METTL3.

Discussion

HCC is an interactive multi-step process, including genetic, epigenetic and transcriptional changes [29]. In the current study, we found that miR-186-5p prevented HCC progression by targeting METTL3 to regulate the m6A-mediated stabilization of FSTL5 (Fig. 9).

FSTL5 is a member of the follistatin gene family that codes secretory glycoprotein. Our previous studies have found that FSTL5 inhibited the HCC progression by inducing apoptosis and regulating the Wnt/YAP (Yes-associated protein) pathway [30]. YAP/TAZ (transcriptional coactivator with PDZ-binding motif) is a transcription-assisted activation factor that interacts with transcription factors combined with DNA to regulate the genes that participate in HCC proliferation and transformation. Li C et al. suggested that FSTL5 accelerated the HCC cell apoptosis in a caspase-dependent manner [8]. However, what prompts the downregulation of FSTL5 expression in HCC has not yet been reported.

Interestingly, our result indicated that the FSTL5 mRNA t_{1/2} was shortened in HCC cells, suggesting the possible presence of RNA modification. These data inspired us to hypothesize that m6A is involved in regulating the stability of FSTL5 in HCC. Emerging studies reported that epigenetic modifications, such as miRNA regulation, histone modification, and DNA methylation, are critically involved in the pathogenesis and development of HCC [31, 32]. m6A RNA methylation is the most common RNA modification. Various studies have elucidated the effects of m6A modification, including its effect on mRNA stability which functioned in the development of HCC [33, 34]. For instance, Chen M et al. indicated that METTL3 inhibited the expression of SOCS2 (suppressor of cytokine signaling 2) mRNA by mediating the modification of SOCS2 mRNA m6A [35]. METTL3 plays its regulatory role through m6A modification-mediated mRNA stability [36]. We found that METTL3 promoted the FSTL5 mRNA degradation. YTHDF2 is a functional m6A-binding protein that mainly regulates the stability of mRNA based on sufficient verification and research [37]. According to reports, the METTL3/YTHDF2 m6A axis promotes the tumor development by decomposing the SET domain-containing lysine methyltransferase 7 (SETD7) and Krüppel-like factor 4 (KLF4) mRNAs in bladder cancer [38].

Consistently, we found that the overexpression of YTHDF2 promoted the degradation of FSTL5 mRNA to inhibit the FSTL5 expression by recognizing the CDS region of FSTL5 mRNA. In an ongoing study, we found that FSTL5 was lowered in HCC tissues, while METTL3 and YTHDF2 were upregulated in HCC tissues, which indicates the poor prognosis of HCC in the TCGA database. Moreover, METTL3 abolished

the anti-tumor effect of FSTL5, indicating that FSTL5 was regulated by METTL3-dependent m6A modification in HCC. miRNAs participate in various disease processes by regulating the gene expression negatively at the post-transcriptional level [39]. The abnormal regulation of miRNA expression is associated with tumor progression. Cai X et. al indicated that hepatitis B X-interacting protein (HBXIP) promoted the progression of breast cancer by inhibiting miRNA let-7g to regulate METTL3, and let-7g downregulated the METTL3 expression by targeting 3'UTR [40].

However, the studies on whether miRNAs could regulate the m6A process in HCC development remain limited. Herein, we screened the miR-186-5p by taking the intersection of online databases Targetscan, miRWalk, and Starbase. The RT-qPCR assay showed that miR-186-5p was significantly downregulated in HCC tissues. An in vitro study on the effects of miR-186-5p on HCC cells further revealed that it inhibits the proliferation, invasion, and migration of HCC cells. We hypothesize that miR-186-5p may regulate the m6A modification to increase the stability or decrease the degradation of the FSTL5 level. Then, we detected the m6A level in miR-186-5p-treated HCC cells and found that the m6A level decreased with miR-186-5p overexpression. We found that miR-186-5p negatively regulated the m6A level by directly targeting the METTL3 in HCC cells. The overexpression of METTL3 abolished the miR-186-5p's anti-tumor effect, indicating that miR-186-5p's effect was dependent on the downregulation of METTL3.

In conclusion, we identified the downregulated FSTL5 in HCC and the m6A-regulated FSTL5 stability mediated by YTHDF2. We reported the crosstalk of miRNA with m6A-mediated protein expression. The miR-186-5p/METTL3/YTHDF2/FSTL5 axis may provide a new idea for the targeted therapy of HCC.

Abbreviations

HCC

Hepatocellular carcinoma; IHC:Immunohistochemistry; miRNA:microRNA; m6A:N6-methyladenosine; FSTL5:Follistatin-like 5; YTHDF2:YTH domain family 2; METTL3:methyltransferase-like 3; TCGA:the cancer genome atlas; ATCC:American Type Culture Collection; DMEM:Dulbecco's modified Eagle's medium; FBS:fetal bovine serum; shRNA:short hairpin RNA; IGF2BP:insulin-like growth factor 2 mRNA-binding protein 2; HE:Hematoxylin and eosin; ECL:Enhanced chemiluminescence; RIPA:Radio-Immunoprecipitation Assay; CCK-8:Cell Counting Kit-8; RT-qPCR:Reverse Transcription Polymerase Chain Reaction; hnRNPA2B1:heterogeneous nuclear ribonucleoprotein A2B1; METTL14:Methyltransferase-like 14; ALKBH5:AlkB homolog 5; FTO:Fat mass and obesity-associated protein; SOCS2:suppressor of cytokine signaling 2; YAP:Yes-associated protein; TAZ:transcriptional coactivator with PDZ-binding motif; KLF4:Krüppel-like factor 4; SETD7:SET domain-containing lysine methyltransferase 7; HBXIP:hepatitis B X-interacting protein.

Declarations

Acknowledgements

Not applicable

Authors' contributions

DengYong Zhang completed most of the experiments in vitro; ShuoShuo Ma and FangFang Chen completed the in vivo assays and helped to draft the manuscript; Yong Chun Zhou and WanLiang Sun collected the tissue samples and help to analysed the experimental data; DongDong Wang and ShiRu Chang finished the data collection and analysition; Zheng Lu guided the thoughts of the study and amended the manuscript. All authors read and approved the final manuscript.

Funding

This study was supported by Anhui Province Science Foundation for Youths (1808085QH288), Bengbu Medical College Science Foundation (BYKY2019116ZD).

Availability of data and materials

The prognosis of the indicated patients' cohort were obtained from TCGA (<https://www.cancer.gov/tcga>). The processed data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Bengbu Medical College (No. 2017059).

Consent for publication

All authors agree to publish this article.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

FSTL5 inhibits HCC cell proliferation, migration, and invasion. (A) The RT-qPCR assay showed the significantly decreased expression of FSTL5 in HCC tissues. n = 28. (B) The IHC assay indicated the

downregulated FSTL5 level in HCC tissues. Bar = 20 μ m. (C) The RT-qPCR and (D) western blot assays indicated that FSTL5 expression was downregulated in HCC cell lines Huh-7, MHCC97-H, HepG2, and SMMC-7721. (E) The transfection efficiency of the FSTL5 overexpression plasmid (FSTL5 OE) was confirmed by RT-qPCR assay. (F) Cell viability, (G) clone formation, (H) migration, and invasion were inhibited by FSTL5 overexpression. Bar = 100 μ m. The data represent three separate experiments and are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with the control group.

Figure 2

METTL3 promotes FSTL5 mRNA degradation. (A) The RNA stability assay showed the FSTL5 mRNA half-life ($t_{1/2}$) in HepG2 cells. (B) The total m6A level of adjacent non-tumor tissues and tumor tissues. n = 28. (C) The METTL3, METTL14, FTO, and ALKBH5 levels were detected in HCC tissues by RT-qPCR. (D) The MeRIP qPCR assay was performed to assess the effect of METTL3 targeting the FSTL5 mRNA on m6A modification. (E) The RNA stability assay showed the FSTL5 mRNA half-life ($t_{1/2}$) in HepG2 cells with METTL3 knockdown. (F) RT-qPCR and (G) western blot assays showed that METTL3 negatively regulated the FSTL5 expression. (H) Luciferase reporter assay showed the relationship between METTL3 and FSTL5. (I) RT-qPCR and (J) IHC assays indicated that the expression of METTL3 was upregulated in HCC tissues. n = 28. Bar = 20 μ m. (K) Pearson's correlation assay showed the negative correlation between the METTL3 and FSTL5 expression in HCC. The data represent three separate experiments and are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, compared with the control group.

Figure 3

YTHDF2 promotes FSTL5 mRNA degradation in a m6A-dependent manner. (A) The expression of FSTL5 and GAPDH protein was detected by western blot assay. (B) RIP-RT-PCR, followed by agarose gel electrophoresis, showed the binding of FSTL5 with YTHDF2. (C) After actinomycin D (5 mg/mL) treatment, the RNA stability assay showed the FSTL5 mRNA half-life ($t_{1/2}$) of HepG2 cells overexpressed with YTHDF2. (D) YTHDF2 was pulled down by full length and wild type of the CDS region of FSTL5 mRNA. (E) IHC staining showed the YTHDF2 expression in adjacent non-tumor and tumor tissues. Bar = 20 μ m. (F) The results based on the TCGA database showed the correlation of METTL3 and YTHDF2 expressions with the prognosis of HCC patients in the TCGA cohort. The data represent three separate experiments and are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

Figure 4

Downregulated METTL3 inhibits HCC cell proliferation, migration, and invasion by up-regulating FSTL5. (A) The transfection efficiency of sh-METTL3-1 and sh-FSTL5-1 in Huh-7 and HepG2 cells was confirmed by RT-qPCR assay. (B) CCK-8 and (C) colony formation assays were used to detect cell proliferation. (D) Transwell assay was performed to detect cell migrations and invasion. Bar = 100 µm. The data represent three separate experiments and are expressed as mean ± SEM. *** $p < 0.001$, * $p < 0.05$, ** $p < 0.01$.

Figure 5

Downregulated METTL3 inhibits HCC tumor growth by up-regulating FSTL5 in vivo. A tumor xenograft model was constructed through the subcutaneous injection of 1×10^7 HepG2 cells. The knockdown of METTL3 and FSTL5 was performed using the METTL3 knockdown lentivirus and FSTL5 knockdown lentivirus (GenePharma, Shanghai, China), respectively, by the intratumoral injection of 50 μ L of virus (4×10^7 IU/mL) after the tumor cell injection. (A) Pictures of the isolated tumors of the indicated group. $n = 5$.

(B) The tumor of mice in the indicated group is shown. n = 5. (C) The expression of METTL3, FSTL5, and GAPDH in the tumor tissues were detected by western blot assay. (D) HE and IHC staining were used to assess tumor infiltration and Ki67 expression. Bar = 200 μ m. The data represent three separate experiments and are expressed as mean $\pm$ SEM. ***p < 0.001, *p < 0.05, **p < 0.01.

Figure 6

MiR-186-5p inhibits HCC cell proliferation, migration, and invasion. (A) The correlation between the METTL3 protein and mRNA expression in HCC tissues is shown. n = 28. (B) Online databases Targetscan, miRWalk, and Starbase were used to predict the possible miRNAs. (C) The miR-186-5p level in HCC tissues was confirmed by RT-qPCR assay. n = 28. miR-186-5p was overexpressed or knocked down in Huh-7 and HepG2 cells by treating the cells with miR-186-5p mimic or inhibitor for 24 h. (D) The transfection efficiency was verified by RT-qPCR. (E) Clone formation was inhibited by miR-186-5p overexpression and increased miR-186-5p knockdown cells. (F) Transwell assay showed that cell invasion and migration were inhibited by miR-186-5p overexpression and increased miR-186-5p

knockdown cells. Bar = 100 μ m. The data represent three separate experiments and are expressed as mean $\pm$ SEM. *** $p < 0.001$, * $p < 0.05$, ** $p < 0.01$.

Figure 7

METTL3 is a direct target of miR-186-5p. (A) The HepG2 cells were treated with miR-186-5p mimic or inhibitor for 24 h. The total m6A level in the cells of the indicated group is shown. (B) The METTL3, METTL14, ALKBH5, FTO, and GAPDH protein expression levels were detected by western blot assay. (C) The potential binding sites of miR-186-5p and 3'UTR of METTL3 mRNA. (D) Luciferase reporter assay was performed for 48 h, with luciferase reporter plasmid containing the wild type or mutant METTL3 3' UTR and NC, miR-186-5p mimic, and miR-186-5p inhibitor. (E) The protein expression of FSTL5 was assessed by western blot assay. (F) MeRIP qPCR assay was used to evaluate the m6A level of FSTL5 mRNA. The data represent three separate experiments and are expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 8

MiR-186-5p inhibits HCC cell proliferation, migration, and invasion by downregulating METTL3. The Huh-7 and HepG2 cells were treated with miR-186-5p mimic, with or without transfection with METTL3 overexpression lentivirus for 24 h. (A) The cell viability and (B) clone formation were inhibited by miR-186-5p overexpression, which was reversed by METTL3 overexpression. (C) Transwell assay showed that cell invasion and migration were inhibited by miR-186-5p overexpression, which was reversed by METTL3 overexpression. Bar = 100 μ m. The data represent three separate experiments and are expressed as mean $\pm$ SEM. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 9

The mechanism of the regulatory network miR-186-5p/METTL3/YTHDF2/FSTL5 axis.

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