ORIGINAL RESEARCH N⁶-Methyladenosine-Modified ATP8B1-AS1 Exerts Oncogenic Roles in Hepatocellular Carcinoma via Epigenetically Activating MYC

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Purpose: N⁶-methyladenosine (m⁶A) modification has shown critical roles in regulating mRNA fate. Non-coding RNAs also have important roles in various diseases, including hepatocellular carcinoma (HCC). However, the potential influences of m⁶A modification on non-coding RNAs are still unclear. In this study, we identified a novel m⁶A-modified ATP8B1-AS1 and aimed to investigate the effects of m⁶A on the expression and role of ATP8B1-AS1 in HCC.

Methods: qPCR was performed to measure the expression of related genes. The correlation between gene expression and prognosis was analyzed using public database. m⁶A modification level was measured using MeRIP and single-base elongation- and ligationbased qPCR amplification method. The roles of ATP8B1-AS1 in HCC were investigated using in vitro and in vivo functional assays. The mechanisms underlying the roles of ATP8B1-AS1 were investigated by ChIRP and ChIP assays.

Results: ATP8B1-AS1 is highly expressed in HCC tissues and cell lines. High expression of ATP8B1-AS1 is correlated with poor overall survival of HCC patients. ATP8B1-AS1 is m⁶A modified and the 792 site of ATP8B1-AS1 is identified as an m⁶A modification site. m⁶A modification increases the stability of ATP8B1-AS1 transcript. m⁶A modification level of ATP8B1-AS1 is increased in HCC tissues and cell lines, and correlated with poor overall survival of HCC patients. ATP8B1-AS1 promotes HCC cell proliferation, migration, and invasion, which were abolished by the mutation of m⁶A-modified 792 site. Mechanistic investigation revealed that m⁶A-modified ATP8B1-AS1 interacts with and recruits m⁶A reader YTHDC1 and histone demethylase KDM3B to MYC promoter region, leading to the reduction of H3K9me2 level at MYC promoter region and activation of MYC transcription. Functional rescue assays showed that depletion of MYC largely abolished the oncogenic roles of ATP8B1-AS1.

Conclusion: m⁶A modification level of ATP8B1-AS1 is increased and correlated with poor prognosis in HCC. m⁶A-modified ATP8B1-AS1 exerts oncogenic roles in HCC via epigenetically activating MYC expression.

Keywords: hepatocellular carcinoma, N⁶-methyladenosine, noncoding RNA, histone methylation, MYC signaling

Introduction

Liver cancer is one of the most common malignancies worldwide, which has relatively poor prognosis.¹ According to global cancer statistics 2020, liver cancer is the six most commonly diagnosed cancer, following breast, lung, colorectal, prostate, and stomach cancers.² However, liver cancer is the third leading cause of cancer death, following lung and colorectal cancers.² Hepatocellular carcinoma (HCC) is the major histological subtype of liver cancer.³ Until now, the efficient therapy for HCC is still surgical resection.⁴ Unlike other common cancers, such as lung and breast cancers, liver cancer is not sensitive to commonly used targeted therapy and immunotherapy.^{5–8} Thus, further revealing the molecular mechanisms underlying HCC initiation and progression is urgently needed.

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Many molecules and signaling pathways aberrations have been found in HCC, such as P53, Wnt–β-catenin signaling, TGF-β signaling, IL6–JAK–STAT signaling, AKT signaling, MET signaling, MYC signaling, and et al.^{9–13} Underlying these signaling pathways, many genetic and epigenetic aberrations have been identified.¹⁴ Classical genetic aberrations found in HCC include chromosome 17p loss, *TP53* mutation, *TERT* promoter mutation, *CTNNB1* mutation, *AXIN1* mutation.³ Compared with genetic aberrations, more epigenetic aberrations have also been identified in HCC, such as DNA methylation, histone acetylation, and histone methylation.¹⁵ Aberrant epigenetic modifications regulate the expression of target genes, which contributes to more frequently found gene expression dysregulations in HCC.¹⁶

Recently, apart from epigenetic modifications, epitranscriptomic modifications also have been shown to play important roles in gene expression modulation.¹⁷ Among epitranscriptomic modifications, N⁶-methyladenosine (m⁶A) modification is the most common internal modification in eukaryotic RNA.¹⁸ m⁶A is reversibly added by writers, such as the methyltransferases METTL3, METTL14, WTAP, and METTL16, and removed by erasers, such as the demethylases FTO and ALKBH5.^{19–22} m⁶A-modified RNAs were recognized by readers, such as YTHDC1/2, YTHDF1/2/3, IGF2BP1/2/3, and HNRNPG/C.^{23,24} m⁶A has critical roles in RNA metabolisms and fates, such as RNA splicing, nuclear export, translation, and stability.²⁵ Until now, most m⁶A modifications were found in messenger RNAs (mRNAs).²⁶ Long noncoding RNA (lncRNA) is another class of long RNAs with limited protein-encoding potential.^{27–30} Dysregulated expressions and critical roles of lncRNAs in various diseases, including cancers have been frequently revealed.^{31–36} Several studies have revealed m⁶A modification in lncRNAs.^{37,38} METTL3 installed m⁶A modification in lncKCNQ10T1, which increased the stability and expression of lncKCNQ10T1.³⁷ However, METTL16-induced m⁶A modification in lncRNA RAB11B-AS1 decreased the stability and expression of RAB11B-AS1.³⁸ The other potential influences of m⁶A modification on the fates of lncRNAs need further investigation.

A previous study has identified several m⁶A modification and prognosis-related lncRNAs in HCC via analyzing The Cancer Genome Atlas (TCGA) Liver Hepatocellular Carcinoma (LIHC) data.³⁹ Among these lncRNAs, we found that not only the expression level of ATP8B1-AS1 (also annotated as AC027097.1 or RP11-35G9.3), but also the m⁶A modification level of ATP8B1-AS1 was increased in HCC and associated with poor prognosis of HCC patients. Thus, we focused on ATP8B1-AS1 in this study. *ATP8B1-AS1* is located in chromosome 18q21.31. *ATP8B1-AS1* has 3 exons and its antisense strand is *ATP8B1*. In this study, we further investigated the contribution of m⁶A modification to the expression, role, and clinical relevance of ATP8B1-AS1 in HCC. We identified the classical oncogene *MYC* as the critical downstream target of m⁶A-modified ATP8B1-AS1. MYC has been shown to be increased and play oncogenic roles in HCC.^{40,41}

Materials and Methods

HCC Samples

The correlation between ATP8B1-AS1 expression and overall survival of HCC patients based on TCGA-LIHC data was analyzed using the online tool GEPIA (<u>http://gepia.cancer.pku.cn/</u>). The TCGA-LIHC RNA-seq data and corresponding clinical information were downloaded from <u>https://portal.gdc.cancer.gov/</u>. Furthermore, 79 pairs of HCC tissues and matched adjacent noncancerous liver tissues were randomly obtained from HCC patients who received surgery at the Affiliated Hospital of Youjiang Medical University for Nationalities. Written informed consents were acquired from all participants. This study was performed following the Helsinki Declaration and with the approval from Youjiang Medical University for Nationalities Institutional Review Board.

Cell Culture

Human immortalized liver cell-line THLE-2 (cat. no. CRL-2706) and HCC cell line SNU-398 (cat. no. CRL-2233) were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). Human HCC cell lines HuH-7 (cat. no. SCSP-526) and SK-HEP-1 (cat. no. TCHu109) were acquired from the Chinese Academy of Sciences Cell Bank (Shanghai, China). THLE-2 was maintained in the BEGM Bullet Kit (cat. no. CC-3170, Lonza). SNU-398 was maintained in RPMI 1640 medium (Invitrogen Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen). HuH-7 was cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from indicated tissues and cells using the RNA isolater Total RNA Extraction Reagent (cat. no. R401, Vazyme, Nanjing, China). The RNA was subjected to reverse transcription using the HiScript III RT SuperMix for qPCR (cat. no. R323, Vazyme) to generate the first strand complementary DNA (cDNA). The cDNA was further subjected to quantitative polymerase chain reaction (qPCR) using the ChamQ Universal SYBR qPCR Master Mix (cat. no. Q711, Vazyme) on QuantStudio Real-Time PCR Instrument (Applied biosystems, Foster City, CA, USA) with the primers 5'-AGTTTCACTCTTGTTGCCC-3' (sense) and 5'-TCTTGTCCCTCTGTCCTT-3' (antisense) for ATP8B1-AS1, 5'-TGCTGTGGCAGAAAAGAAG-3' (sense) and 5'-TCAGACCAGAGACTAACGAAC-3' (antisense) for METTL3, 5'-ACTGTGGGAAGAAGAAGAAGGAGG-3' (sense) and 5'-TGAAACGATGTCTGTGAGGT-3' (antisense) for FTO, 5'-CTTCCCCTACCTCTCAA-3' (sense) and 5'-CGATTTCTTCCTCATCTTCT-3' (antisense) for MYC, 5'-ATCAAGTAGTGCCTCCAG-3' (sense) and 5'-CCATCACCATCTCCTCAG-3' (antisense) for YTHDC1, 5'-GCCTCCAACAAAAACC-3' (sense) and 5'-CCATCACCATCTCCTCAC-3' (antisense) for KDM3B, 5'-GTCGGAGTCAACGGATTTG-3' (sense) and 5'-TGGGTGGAATCATATTGGAA-3' (antisense) for GAPDH. GAPDH was used as endogenous control. Relative quantification was calculated using the comparative Ct method.

Western Blot

Total protein was isolated from indicated cells using the RIPA Lysis Buffer (Beyotime, Shanghai, China), which was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by being transferred onto nitrocellulose membrane. After block using 5% nonfat milk, the membranes were incubated with primary antibodies specific for METTL3 (cat. no. ab195352, 1:1000, Abcam, Cambridge, MA, USA), FTO (cat. no. ab126605, 1:1000, Abcam), MYC (cat. no. ab32072, 1:1000, Abcam), or GAPDH (cat. no. ab8245, 1:5000, Abcam). The second antibodies used were Goat anti-Mouse IgG H&L (IRDye® 680RD) preadsorbed (cat. no. ab216776, 1:10,000, Abcam) or Goat anti-Rabbit IgG H&L (IRDye® 800CW) preadsorbed (cat. no. ab216773, 1:10,000, Abcam). Lastly, the membranes were scanned on an Odyssey infrared scanner ((Li-Cor, Lincoln, NE, USA).

Vectors Construction, Transfection, and Stable Cell Lines Construction

The cDNA encoding ATP8B1-AS1 was PCR-amplified with the PrimeSTAR Max DNA Polymerase (cat. no. R045Q, Takara, Tokyo, Japan) and the primers 5'-TTGGTACCGAGCTCGGATCCATTTCCTACTTGGTCCTGTTC-3' (sense) and 5'-GTGCTGGATATCTGCAGAATTCTTCTAAGCAGAATTGAAATTTTTAT-3' (antisense), followed by being cloned into the BamH I and EcoR I sites of the pcDNA3.1(+) vector (Invitrogen) using the NovoRec plus One step PCR Cloning Kit (cat. no. NR005, Novoprotein, Suzhou, China) to construct ATP8B1-AS1 expression vector. The m⁶A-modified 792 site mutated ATP8B1-AS1 expression vector was constructed using the Fast Mutagenesis System (cat. no. FM111, TransGen, Beijing, China) with the primers 5'-TCAAAGCACTGGAAGGTC AGAGGGACAA-3' (sense) and 5'-ACCTTCCAGTGCTTTGACATTTGGTGGG-3' (antisense). Two pairs of cDNA oligonucleotides targeting ATP8B1-AS1 were synthesized and cloned into the shRNA lentivirus expressing vector (GenePharma, Shanghai, China) to generate shRNA lentivirus targeting ATP8B1-AS1. Scrambled non-targeting shRNA lentivirus were used as negative control (NC). The sequences of shRNA oligonucleotides were as follows: 5'-GATCCGCAAAGGAAACGGCAGCTATATTCAAGAGATATAGCTGCCGTTTCCTTTGCTTTTTTG-3' (sense) and 5'-AATTCAAAAAAGCAAAGGAAACGGCAGCTATATCTCTTGAATATAGCTGCCGTTTCCTTTGCG-3' (antisense) for shRNA-ATP8B1-AS1-1, 5'-GATCCGCTGCCATGTTTCACAATTACTTCAAGAGAGTAATTGTGAAA CATGGCAGCTTTTTTG-3' (sense) and 5'-AATTCAAAAAAGCTGCCATGTTTCACAATTACTCTCTTGAAGTAA TTGTGAAACATGGCAGCG-3' (antisense) for shRNA-ATP8B1-AS1-2, 5'-GATCCGTTCTCCGAACGTGTCACGTT TCAAGAGAACGTGACACGTTCGGAGAACTTTTTTG-3' (sense) and 5'-AATTCAAAAAAGTTCTCCGAACGTG TCACGTTCTTGAAACGTGACACGTTCGGAGAACG-3' (antisense) for shRNA-NC. METTL3 and FTO expression vectors were obtained from GenePharma. ON-TARGETplus Human YTHDC1 siRNA SMART Pool (cat. no.

L-015332-02-0010), ON-TARGETplus Human KDM3B siRNA SMART Pool (cat. no. L-020378-01-0010), and ON-TARGETplus Human MYC siRNA SMART Pool (cat. no. L-003282-02-0010) were purchased from Horizon Discovery (Cambridge, England). The transfection of vectors and siRNAs was conducted using the GP-transfect-Mate (GenePharma).

To construct HCC cells with wild-type or m⁶A-modified 792 site mutated ATP8B1-AS1 stable overexpression, ATP8B1-AS1 expression vector or m⁶A-modified 792 site mutated ATP8B1-AS1 expression vector was transfected into SNU-398 and HuH-7 cells. Forty-eight hours later, the cells were treated with 1000 μ g/mL neomycin for four weeks to select cells with stable wild-type or m⁶A-modified 792 site mutated ATP8B1-AS1 overexpression. To construct HCC cells with ATP8B1-AS1 stable depletion, shRNA lentivirus targeting ATP8B1-AS1 were infected into SNU-398 and SK-HEP-1 cells. Ninety-six hours later, the cells were treated with 2 μ g/mL puromycin for four weeks to select cells with stable ATP8B1-AS1 depletion.

m⁶A Modification Detection

m⁶A-modified transcripts were enriched by the methylated RNA immunoprecipitation (MeRIP) assays using the Magna MeRIP m⁶A Kit (cat. no. 17–10499, Millipore, Billerica, MA, USA). The enriched RNAs were detected by qPCR as described above. The specific m⁶A modification site was predicted by the online tool SRAMP (<u>http://www.cuilab.cn/sramp</u>). m⁶A modification level of 792 site of ATP8B1-AS1 was measured using the previously described single-base elongation- and ligation-based qPCR amplification method (termed "SELECT").⁴² Concurrent detection of non-modified 788 site was carried out and served as input. The sequences of probes used were as follows: 5'-tagcca gtaccgtagtgcgtgGGGCCATCCCTCTTGTCCCTCTG-3' (up) and 5'-5phos/CCTTCCAGTGCTTTGACATTTGGT GCagaggctgagtcgctgcat-3' (down) for 792 site, 5'-tagccagtagtgcgtgcgtgcat-3' (down) for 788 site. The primers for qPCR of SELECT were 5'-ATGCAGCGACTCAGCCTCTG-3' (sense) and 5'-TAGCCAGTACCGTAGTGCGTG-3' (antisense).⁴²

Cell Proliferation, Migration, and Invasion Assays

Cell proliferation was evaluated using Glo cell viability assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. Glo cell viability assay was performed using the CellTiter-Glo Luminescent Cell Viability Assay (cat. no. G7572, Promega, Madison, WI, USA) after culture for 48h as previously reported.⁴³ The luminescence was used to indicate cell viability. EdU incorporation assay was conducted using the Cell-Light EdU Apollo567 In Vitro Kit (cat. no. C10310-1, RiboBio, Guangzhou, China) following the provided protocol. Briefly, after culture for overnight, indicated HCC cells were treated with 50 µM EdU for 2h. Next, the treated cells were fixed in 4% paraformaldehyde for 30 min and permeabilized in 0.5% Triton X-100 for 10 min. The cells were further stained with Apollo dye solution (RiboBio) and the cell nucleuses were stained with DAPI. The number of EdU-positive cells was assessed using the fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Cell migration and invasion were evaluated by transwell migration and invasion assays, respectively. Briefly, indicated cells resuspended in 200µL serum-free medium were loaded in the upper chambers of the Millicell Hanging Inserts (8-µm pore, Millipore) precoated with or without Matrigel (BD Biosciences, San Jose, CA, USA). Complete medium supplemented with 10% FBS was loaded into the lower chambers. After culture for 24h, the cells remaining on the upper chambers were removed. The cells invading into the bottom side of the inserts were fixed, stained, and quantified under a microscope by counting at least five random high-power fields.

Subcutaneous Tumor Growth Assay

Indicated HCC cells were subcutaneously inoculated into the flank of 5-week-old male BALB/C athymic mice, which were purchased from Shanghai SLAC Laboratory Animal Co. and fed in specific pathogen-free condition. Subcutaneous tumor volumes were measured every week and calculated using the formula 0.5×length×width². At the 28th day after inoculation, subcutaneous tumors were resected, weighed, and photographed. This study was approved by Affiliated Hospital of Youjiang Medical University for Nationalities Institutional Review Board.

Chromatin Isolation by RNA Purification (ChIRP) Assay

ChIRP assay was conducted in SNU-398 cells using the EZ-Magna ChIRP RNA Interactome Kit (cat. no. 17–10495, Millipore). The sequences of ATP8B1-AS1 antisense DNA probes were as follows: 1, 5'-ccacgaacaggaccaagtag-3'; 2, 5'- aagcctgttatgaggagcaa-3'; 3, 5'-aattggtcttatctctccag-3'; 4, 5'-ggacggaagatgcttttct-3'; 5, 5'-cattgtgcagggaagcatat-3'; 6, 5'- tgggagctacagctattgaa-3'; 7, 5'-attagcccttctttttacag-3'; 8, 5'-aagtttttaggtgtgtggt-3'; 9, 5'-ggtttacggtttatgtcagt-3'; 10, 5'- tacattgggggaattaccga-3'; 11, 5'-gcagaacagaagtgccatt-3'. The enriched DNA was measured by qPCR with the primers: 5'- CTTCTTTCCTCCACTCTCCC-3' (sense) and 5'-ACCCTCTCCTTCTCTGC-3' (antisense) for *MYC* promoter; 5'- GACGCTTTCTTTCCTTCCGC-3' (sense) and 5'-CTGCCCATTCATTTCCTTCC-3' (antisense) for *GAPDH* promoter.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was conducted in SNU-398 cells using the EZ-Magna ChIP A/G Chromatin Immunoprecipitation Kit (cat. no. 17–10086, Millipore) and a KDM3B antibody (cat. no. 5377, Cell Signaling Technology, Danvers, MA, USA) or an H3K9me2 antibody (cat. no. ab1220, Abcam). The enriched DNA was measured by qPCR with the primers: 5'-CTTCTTTCCTCCACTCTCCC-3' (sense) and 5'-ACCCTCTCCCTTTCTCTGC-3' (antisense) for *MYC* promoter.

Statistical Analysis

The GraphPad Prism v6.0 Software (San Diego, CA, USA) was used to carry out all statistical analyses. Mann–Whitney test, Wilcoxon matched-pairs signed rank test, Log rank test, one-way ANOVA followed by Dunnett's multiple comparisons test, Spearman correlation analysis, or two-tailed unpaired *t*-test were performed as shown in figure legends. P < 0.05 was considered statistically significant.

Results

ATP8B1-AS1 is Highly Expressed and Correlated with Poor Overall Survival in HCC

The clinical correlation of ATP8B1-AS1 in HCC was first analyzed using the TCGA-LIHC data. The results showed that high expression of ATP8B1-AS1 was correlated with short overall survival time (Figure 1A). ATP8B1-AS1 was highly expressed in HCC tissues compared with that in normal liver tissues (Figure 1B). Furthermore, the expression of ATP8B1-AS1 was higher in HCC tissues with poor differentiation than those with good differentiation (Figure 1C). The TCGA-LIHC data also revealed that the expression of ATP8B1-AS1 was positively correlated with high alpha-fetoprotein (AFP) level (Figure 1D). To further investigate the clinical relevance of ATP8B1-AS1 in HCC, we collected 79 pairs of HCC tissues and matched adjacent noncancerous liver tissues and measured ATP8B1-AS1 expression in these tissues. The results showed that ATP8B1-AS1 was also highly expressed in HCC tissues compared with short overall survival time in our HCC cohort (Figure 1F). The expression of ATP8B1-AS1 was correlated with short overall survival time in our HCC cohort (Figure 1F). The expression of ATP8B1-AS1 was correlated with the expression pattern in HCC tissues, ATP8B1-AS1 was also highly expressed in HCC cell lines compared with that in immortalized liver cell line (Figure 1G). Therefore, these data suggested that ATP8B1-AS1 is highly expressed and correlated with poor overall survival in HCC.

m⁶A Modification Increases the Stability of ATP8B1-AS1 Transcript

To investigate the potential correlation between m⁶A modification and ATP8B1-AS1, we first analyzed the correlation between the expression of ATP8B1-AS1 and m⁶A methylases in HCC tissues using the TCGA-LIHC data. The results showed that the expression of ATP8B1-AS1 was positively correlated with the expression of m⁶A methylases METTL3 and WTAP (Figure 2A and B), implying that ATP8B1-AS1 may be modulated by m⁶A modification. RIP assays using m⁶A antibody showed that m⁶A modification of ATP8B1-AS1 was found in all tested HCC cell lines, including HuH-7, SNU-398, and SK-HEP-1 (Figure 2C). The online tool SRAMP (<u>http://www.cuilab.cn/sramp</u>) predicted the 792 site of ATP8B1-AS1 as the very high confident m⁶A modification site (Figure 2D). Next, we used the previously reported single-base elongation- and ligation-based qPCR amplification method (SELECT) to detect the m⁶A modification level of



Figure 1 ATP881-AS1 is upregulated and correlated with poor overall survival in HCC. (**A**) The correlation between ATP881-AS1 (RP11-35G9.3) expression and overall survival in HCC based on TCGA-LIHC RNA-seq data, analyzed by GEPIA. (**B**) ATP881-AS1 expression in HCC tissues (n = 371) and normal liver tissues (n = 50), based on TCGA-LIHC RNA-seq data. (**C**) ATP881-AS1 expression in HCC tissues with grade 1–2 (n = 232) or grade 3–4 (n = 134), based on TCGA-LIHC RNA-seq data. (**D**) ATP881-AS1 expression in HCC tissues with AFP level ≤ 400 (n = 213) or > 400 (n = 65), based on TCGA-LIHC RNA-seq data. For (**B**–**D**), *P* values were calculated by Mann–Whitney test. (**E**) ATP881-AS1 expression in 79 pairs of HCC tissues and matched adjacent liver tissues was measured by qPCR. *P* values were calculated by Wilcoxon matched-pairs signed rank test. (**F**) Kaplan-Meier survival curve of our HCC cohort stratified by ATP881-AS1 expression level. Median ATP881-AS1 expression level was used as cut-off. n = 79, HR = 1.920, *P* = 0.0314 by Log rank test. (**G**) ATP881-AS1 expression in mmortalized liver cell line THLE-2 and HCC cell lines HuH-7, SNU-398, and SK-HEP-1 was detected by qPCR. Results are presented as mean \pm SD based on three independent experiments. *****P* < 0.0001 by one-way ANOVA followed by Dunnett's multiple comparisons test.

792 site of ATP8B1-AS1 (Figure 2E).⁴² The results showed that the 792 site of ATP8B1-AS1 was really m⁶A modified in both HuH-7 and SNU-398 cells (Figure 2F). The m⁶A modification level of 792 site of ATP8B1-AS1 was increased by m⁶A methylase METTL3 and was decreased by m⁶A demethylase FTO in both HuH-7 and SNU-398 cells (Figures <u>S1</u> and F). RNA stability assays showed that ectopic expression of METTL3 elongated the half-life of ATP8B1-AS1 transcript, and while ectopic expression of FTO shortened the half-life of ATP8B1-AS1 transcript in both HuH-7 and SNU-398 cells (Figure 2G and H), suggesting that m⁶A modification increases the stability of ATP8B1-AS1 transcript.



Figure 2 m⁶A modification increases the stability of ATP8B1-AS1 transcript. (**A**) The correlation between ATP8B1-AS1 expression and METTL3 expression, based on TCGA-LIHC RNA-seq data. r = 0.4780, P < 0.0001 by Spearman correlation analysis. (**B**) The correlation between ATP8B1-AS1 expression and WTAP expression, based on TCGA-LIHC RNA-seq data. r = 0.2894, P < 0.0001 by Spearman correlation analysis. (**C**) MeRIP assays using m⁶A specific antibody, followed by qPCR to detect m⁶A-modified ATP8B1-AS1. (**D**) The m⁶A modification site of ATP8B1-AS1, predicted by SRAMP. (**E**) Schematic of SELECT to detect m⁶A modification level (**F**) The m⁶A modification level of ATP8B1-AS1 in METTL3 or FTO overexpressed HuH-7 and SNU-398 cells was detected by SELECT. (**G** and **H**) The stability of ATP8B1-AS1 transcript over time in METTL3 or FTO overexpressed HuH-7 (**G**) and SNU-398 (**H**) cells was detected after blocking new RNA synthesis using α -amaintin (50 µM) and normalized to 18S rRNA (a product of RNA polymerase 1 that is unchanged by α -amaintin). For (**C**) and (**F**–**H**), results are presented as mean ± SD based on three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.001 by one-way ANOVA followed by Dunnet's multiple comparisons test.

m⁶A Modification Level of ATP8B1-AS1 is Increased and Correlated with Poor Survival in HCC

We next measured the m⁶A modification level of ATP8B1-AS1 in 79 pairs of HCC tissues and matched adjacent noncancerous liver tissues using SELECT. The results showed that m⁶A modification level of ATP8B1-AS1 was increased in HCC tissues compared with that in matched adjacent noncancerous liver tissues (Figure 3A). Kaplan-Meier survival analyses showed that increased m⁶A modification level of ATP8B1-AS1 was correlated with short overall survival time in HCC (Figure 3B). Furthermore, m⁶A modification level of ATP8B1-AS1 was positively correlated with ATP8B1-AS1 expression level in these 79 HCC tissues (Figure 3C), supporting the positive regulation of ATP8B1-AS1



Figure 3 m⁶A modification level of ATP8B1-AS1 is upregulated and correlated with poor overall survival in HCC. (**A**) m⁶A modification level of ATP8B1-AS1 in 79 pairs of HCC tissues and matched adjacent liver tissues was detected by SELECT. P < 0.0001 by Wilcoxon matched-pairs signed rank test. (**B**) Kaplan-Meier survival curve of our HCC cohort stratified by m⁶A modification level of ATP8B1-AS1. Median m⁶A modification level of ATP8B1-AS1 was used as cut-off. n = 79, HR = 2.312, P = 0.0056 by Log rank test. (**C**) The correlation between m⁶A modification level of ATP8B1-AS1 and ATP8B1-AS1 expression level in HCC tissues, n = 79, r = 0.4022, P = 0.0002 by Spearman correlation analysis. (**D**) m⁶A modification level of ATP8B1-AS1 in immortalized liver cell line THLE-2 and HCC cell lines HuH-7, SNU-398, and SK-HEP-1 was detected by SELECT. Results are presented as mean \pm SD based on three independent experiments. **P < 0.01, ***P < 0.001 by one-way ANOVA followed by Dunnett's multiple comparisons test.

transcript stability by m^6A modification. m^6A modification level of ATP8B1-AS1 was also increased in HCC cell lines compared with that in immortalized liver cell line (Figure 3D). Consistent with m^6A modification level of ATP8B1-AS1, the expression of m^6A methylase METTL3 was increased, and while the expression of m^6A demethylase FTO was decreased in HCC cell lines compared with that in immortalized liver cell line (Figure S2A and B). Collectively, these data suggested that consistent with the expression level, the m^6A modification level of ATP8B1-AS1 is also increased and correlated with poor survival in HCC.

ATP8B1-AS1 Promotes HCC Cell Proliferation, Migration, and Invasion in an m⁶A Modification Dependent Manner

To investigate the roles of ATP8B1-AS1 in HCC and the potential contribution of m⁶A modification to the roles of ATP8B1-AS1, we stably overexpressed wild-type and the 792 site mutated ATP8B1-AS1 with similar overexpression efficiencies in SNU-398 and HuH-7 cells (Figure 4A and B). Glo cell viability assays showed that ectopic expression of ATP8B1-AS1 increased cell viability of both SNU-398 and HuH-7 cells, which was abolished by the mutation of m⁶A-modified 792 site of ATP8B1-AS1 (Figure 4C and D). EdU incorporation assays further confirmed that ectopic expression of ATP8B1-AS1 promoted cell proliferation of both SNU-398 and HuH-7 cells, which was abolished by the mutation of m⁶A-modified 792 site of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion of both SNU-398 and HuH-7 cells, which were abolished by the mutation of m⁶A-modified 792 site of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion assays showed that ectopic expression of ATP8B1-AS1 promoted cell migration and invasion of both SNU-398 and HuH-7 cells, which were abolished by the mutation of m⁶A-modified 792 site of



Figure 4 ATP881-AS1 exerts oncogenic roles in HCC in an m⁶A dependent manner. (**A** and **B**) ATP881-AS1 expression in SNU-398 (**A**) and HuH-7 (**B**) cells with wild-type or m⁶A-modified 792 site mutated ATP881-AS1 overexpression was measured by qPCR. (**C** and **D**) Cell proliferation of SNU-398 (**C**) and HuH-7 (**D**) cells with wild-type or mutated ATP881-AS1 overexpression was measured by Glo cell viability assay. (**E**) Cell proliferation of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by Glo cell viability assay. (**E**) Cell proliferation of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by Glo cell viability assay. (**E**) Cell proliferative cells. Scale bars = 100 µm. (**F**) Cell migration of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell migration assays. Scale bars = 100 µm. (**G**) Cell invasion of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell invasion assays. Scale bars = 100 µm. (**G**) Cell invasion of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell invasion assays. Scale bars = 100 µm. (**G**) Cell invasion of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell invasion assays. Scale bars = 100 µm. (**G**) Cell invasion of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell invasion assays. Scale bars = 100 µm. (**G**) Cell invasion of SNU-398 and HuH-7 cells with wild-type or mutated ATP881-AS1 overexpression was measured by transwell invasion assays. Scale bars = 100 µm. Results are presented as mean \pm SD based on three independent experiments. **P* < 0.05, ***P* < 0.01, ******P* < 0.0001, by one-way ANOVA followed by Dunnett's multiple comparisons test. **Abbreviation**: ns, not significant.

ATP8B1-AS1 (Figure 4F and G). Therefore, these data suggested that ATP8B1-AS1 promotes HCC cellular proliferation, migration, and invasion in an m⁶A modification dependent manner.

Depletion of ATP8B1-AS1 Repressed HCC Cell Proliferation, Migration, and Invasion

To further confirm the oncogenic roles of ATP8B1-AS1 in HCC, we stably silenced ATP8B1-AS1 expression in SNU-398 and SK-HEP-1 cells (Figure 5A and B). Glo cell viability assays showed that SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 silencing had decreased cell viability compared with control SNU-398 and SK-HEP-1 cells, respectively (Figure 5C and D). EdU incorporation assays also showed that SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 silencing had repressed cell proliferation compared with control SNU-398 and SK-HEP-1 cells (Figure 5E). Subcutaneous xenograft assays showed that SNU-398 cells with ATP8B1-AS1 silencing formed smaller tumors and had slower tumor growth than control SNU-398 cells (Figure 5F), suggesting that depletion of ATP8B1-AS1 also repressed HCC tumor growth in vivo. Transwell migration and invasion assays showed that SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 silencing had reduced cell migration and invasion compared with control SNU-398 and SK-HEP-1 cells (Figure 5G and H). Therefore, these data suggested that depletion of ATP8B1-AS1 repressed HCC cell proliferation, migration, and invasion.

m⁶A-Modified ATP8B1-AS1 Epigenetically Activates MYC Expression via Decreasing H3K9me2 Level at MYC Promoter

To reveal the mechanisms underlying the oncogenic roles of ATP8B1-AS1 in HCC, we performed Gene Set Enrichment Analysis (GSEA) using the TCGA-LIHC data. According to ATP8B1-AS1 expressing level, the TCGA-LIHC samples were divided into ATP8B1-AS1 high expression group and ATP8B1-AS1 low expression group. GSEA showed that genes from liver cancer subclass S2 were significantly enriched in ATP8B1-AS1 high expression group (Figure 6A). Liver cancer subtype S2 indicates HCC with proliferation, MYC and AKT1 activation.⁴⁴ GSEA of the TCGA-LIHC data further showed that the genes bound by MYC were also significantly enriched in ATP8B1-AS1 high expression group (Figure 6B). Genes upregulated by MYC were significantly enriched in ATP8B1-AS1 high expression group, and while genes downregulated by MYC were significantly enriched in ATP8B1-AS1 high expression group, and while genes downregulated by MYC were significantly enriched in ATP8B1-AS1 high expression group. Thus, the TCGA-LIHC data suggested that ATP8B1-AS1 may be positively correlated with MYC signaling activation.

Next, we investigated the potential influence of ATP8B1-AS1 on MYC. The TCGA-LIHC RNA-data revealed positive correlation between the expression of ATP8B1-AS1 and MYC (Figure 7A). Consistent with m⁶A modification level and the expression of ATP8B1-AS1, the expression of MYC was also increased in HCC cell lines compared with that in immortalized liver cell line (Figure S3A). Ectopic expression of ATP8B1-AS1 upregulated MYC mRNA and protein levels, which were abolished by the mutation of m⁶A-modified 792 site of ATP8B1-AS1 (Figure 7B and S3B). Conversely, silencing of ATP8B1-AS1 downregulated MYC mRNA and protein levels (Figure 7C and S3C). These findings suggested that ATP8B1-AS1 may regulate MYC transcription in an m⁶A modification dependent manner. Recently, m⁶A modified RNA was revealed to bind m⁶A reader YTHDC1, which further binds and recruits the histone H3 lysine 9 di-methylation (H3K9me2) demethylase KDM3B to target gene regions, inducing H3K9me2 demethylation and gene activation.⁴⁵ H3K9me2 is strongly associated with transcriptional repression.⁴⁶ In order to investigate whether m⁶A modified ATP8B1-AS1 regulates MYC expression in such a manner, we first detected whether ATP8B1-AS1 binds MYC promoter using ChIRP assays. The results showed that ATP8B1-AS1 specifically bound to MYC promoter region (Figure 7D). Next, we performed ChIP assays to investigate whether ATP8B1-AS1 influences the binding of KDM3B to MYC promoter region and the H3K9me2 level at MYC promoter region. The results showed that the ectopic expression of ATP8B1-AS1 promoted the binding of KDM3B to MYC promoter region and reduced the H3K9me2 level at MYC promoter region, which were both abolished by the mutation of m⁶A-modified 792 site of ATP8B1-AS1 (Figure 7E). Silencing of ATP8B1-AS1 reduced the binding of KDM3B to MYC promoter region and increased the H3K9me2 level at MYC promoter region (Figure 7F). Depletion of YTHDC1 abolished the increased binding of KDM3B to MYC promoter region, and the reduced H3K9me2 level at MYC promoter region (Figures S3D and 7G), which suggested that the regulation of these epigenetic modifications at MYC promoter by m⁶A-modified ATP8B1-AS1 is dependent on the m⁶A reader YTHDC1. Consistently, depletion of YTHDC1 abolished the upregulation of MYC



Figure 5 Depletion of ATP8B1-AS1 exerts tumor suppressive roles in HCC. (**A** and **B**) ATP8B1-AS1 expression in SNU-398 (**A**) and SK-HEP-1 (**B**) cells with ATP8B1-AS1 depletion was measured by qPCR. (**C** and **D**) Cell proliferation of SNU-398 (**C**) and SK-HEP-1 (**D**) cells with ATP8B1-AS1 depletion was measured by Glo cell viability assay. (**E**) Cell proliferation of SNU-398 (**C**) and SK-HEP-1 (**D**) cells with ATP8B1-AS1 depletion was measured by Glo cell viability assay. (**E**) Cell proliferation of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by EdU incorporation assays. Red color represents EdU-positive and proliferative cells. Scale bars = 100 µm. (**F**) Tumor volume, weight, and photograph of subcutaneous tumors formed by SNU-398 cells with ATP8B1-AS1 depletion. (**G**) Cell migration of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 cells with ATP8B1-AS1 depletion was measured by transwell migration assays. Scale bars = 100 µm. (**H**) Cell invasion of SNU-398 and SK-HEP-1 (**B**) or n=5 mice in each group (**F**). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ****P* < 0.001 µm. Pointer the assays and the point assays and the point assays assays and the point of SNU-398 and SK-HEP-1 (**B**) or mann-Whitney test (**F**).



Figure 6 GSEA shows the correlation between ATP8B1-AS1 expression and MYC signaling activation. TCGA-LIHC samples were divided into ATP8B1-AS1 high expression and low expression group according to median ATP8B1-AS1 expression level. GSEA shows the positive correlation between ATP8B1-AS1 high expression and liver cancer subclass S2 gene signatures (**A**). MYC-bound genes were enriched in ATP8B1-AS1 high expression group (**B**). Genes upregulated by MYC were enriched in ATP8B1-AS1 high expression group, and genes downregulated by MYC were enriched in ATP8B1-AS1 low expression group (**C**). **Abbreviation**: NES, normalized enrichment score.

by m⁶A-modified ATP8B1-AS1 (Figure 7H). Depletion of KDM3B also abolished the upregulation of MYC by m⁶A-modified ATP8B1-AS1 (Figures S3E and 7I). Therefore, these data suggested that m⁶A-modified ATP8B1-AS1 epigenetically activates *MYC* expression via binding YTHDC1 and further interacting with and recruiting KDM3B to decrease H3K9me2 level at *MYC* promoter.

Depletion of MYC Reverses the Oncogenic Roles of ATP8B1-AS1

To assess the potential contribution of MYC to the oncogenic roles of ATP8B1-AS1 in HCC, we silenced MYC expression in ATP8B1-AS1 stably overexpressed SNU-398 cells (Figure 8A). Glo cell viability assays showed that silencing of MYC reversed the increased cell viability caused by ATP8B1-AS1 overexpression (Figure 8B). EdU incorporation assays further showed that silencing of MYC reversed the increased cell proliferation caused by ATP8B1-AS1 overexpression (Figure 8C). Transwell migration and invasion assays showed that silencing of MYC reversed the increased cell migration and invasion caused by ATP8B1-AS1 overexpression (Figure 8D and E). Therefore, these data suggested that the oncogenic roles of ATP8B1-AS1 are dependent on the activation of MYC.

Discussion

As importantly functional molecules in HCC, lncRNAs have been intensively investigated.^{47–49} Many HCC-related lncRNAs show aberrant expression and correlation with prognosis of HCC patients.^{50–52} However, the contribution of m⁶A modification to the expression and role of lncRNAs are still largely unclear.

In this study, we identified ATP8B1-AS1 as an m⁶A-related and prognosis-related lncRNA in HCC. High expression level of ATP8B1-AS1 was previously reported to be correlated with good prognosis in pancreatic adenocarcinoma and breast cancer, but with poor prognosis in HCC.^{39,53,54} However, the potential influences of m⁶A on the clinical significances of ATP8B1-AS1 are still unknown. Here, using public TCGA-LIHC data and our own cohort, we found ATP8B1-AS1 was increased in HCC tissues and cell lines and correlated with poor overall survival of HCC patients. Second, we identified ATP8B1-AS1 as an m⁶A-modified lncRNA and further confirmed the m⁶A modification site on ATP8B1-AS1. Third, we found that the m⁶A modification increased ATP8B1-AS1 was increased in HCC tissues and



Figure 7 ATP881-AS1 epigenetically activates *MYC* expression in an m⁶A dependent manner: (**A**) The correlation between ATP881-AS1 and MYC expression, based on TCGA-LIHC RNA-seq data. r = 0.3206, P < 0.0001 by Spearman correlation analysis. (**B**) MYC expression in SNU-398 cells with wild-type or m⁶A-modified 792 site mutated ATP881-AS1 overexpression was measured by qPCR. (**C**) MYC expression in SNU-398 cells with ATP881-AS1 depletion was measured by qPCR. (**D**) ChIRP assays with ATP881-AS1 antisense probes or control probes were conducted in SNU-398 cells to measure the binding of ATP881-AS1 to *MYC* promoter or *GAPDH* promoter. *GAPDH* promoter was used as negative control. (**E**) ChIP assays with KDM3B or H3K9me2 specific antibodies were conducted in SNU-398 cells with wild-type or m⁶A-modified 792 site mutated ATP881-AS1 overexpression to measure the binding of KDM3B to *MYC* promoter or *GAPDH* promoter. *GAPDH* promoter was used as negative conducted in SNU-398 cells with KDM3B or H3K9me2 specific antibodies were conducted in SNU-398 cells with wild-type or m⁶A-modified 792 site mutated ATP881-AS1 overexpression to measure the binding of KDM3B to *MYC* promoter and H3K9me2 level at *MYC* promoter. (**F**) ChIP assays with KDM3B or H3K9me2 specific antibodies were conducted in SNU-398 cells with ATP881-AS1 depletion to measure the binding of KDM3B to *MYC* promoter and H3K9me2 level at *MYC* promoter. (**G**) ChIP assays with KDM3B or H3K9me2 specific antibodies were conducted in SNU-398 cells with wild-type ATP881-AS1 overexpression and YTHDC1 silencing to measure the binding of KDM3B to *MYC* promoter. (**H**) MYC expression in SNU-398 cells with wild-type ATP881-AS1 overexpression and YTHDC1 silencing was measured by qPCR. (**I**) MYC expression in SNU-398 cells with wild-type ATP881-AS1 overexpression and YTHDC1 silencing was measured by qPCR. (**I**) MYC expression in SNU-398 cells with wild-type ATP881-AS1 overexpression and YTHDC1 silencing was measured by qPCR. (**I**) MYC expression in SNU-398



Figure 8 Depletion of MYC reverses the oncogenic roles of ATP8B1-AS1 in HCC. (**A**) MYC expression in SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by qPCR. (**B**) Cell proliferation of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by Glo cell viability assay. (**C**) Cell proliferation of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by EdU incorporation assays. Red color represents EdU-positive and proliferative cells. Scale bars = 100 μ m. (**D**) Cell migration of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell migration assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. (**E**) Cell invasion of SNU-398 cells with ATP8B1-AS1 overexpression and MYC silencing was measured by transwell invasion assays. Scale bars = 100 μ m. Results are presented as mean ± SD based on three independent experiments. **P < 0.01, ***P < 0.001, by one-way ANOVA followed by Dunnett's multiple comparisons test.

Abbreviation: ns, not significant.

cell lines, and also correlated with poor overall survival of HCC patients. Moreover, the hazard ratio (HR) of m⁶A modification level of ATP8B1-AS1 is greater than the HR of ATP8B1-AS1 expression level, calculated by the same HCC cohort. Thus, these findings suggested that m⁶A modification level of lncRNAs may have more important clinical relevance than the expression of lncRNAs.

Previous reports have showed that m⁶A modification could regulate the splicing, stability, or translation of target mRNAs.^{55,56} m⁶A modification increased the stability of lncKCNQ1OT1, and while decreased the stability of RAB11B-AS1.^{37,38} In this study, we also found that m⁶A modification increases the stability of ATP8B1-AS1. The detailed mechanisms underlying these different effects of m⁶A modification on transcript stability need further investigation.

Furthermore, in this study, we found that m⁶A modification modulates the function of ATP8B1-AS1. ATP8B1-AS1 shows oncogenic roles in HCC, including promoting HCC cell proliferation, migration, and invasion. The oncogenic roles of ATP8B1-AS1 in HCC are dependent on m⁶A modification, as mutation of the m⁶A modification site nearly completely abolished the oncogenic roles of ATP8B1-AS1.

Considering the importance of m⁶A modification in the function of ATP8B1-AS1, we hypothesized that the mechanisms of ATP8B1-AS1 are also dependent on m⁶A modification. Through performing GSEA of TCGA-LIHC data, we identified MYC signaling as the critical downstream target of ATP8B1-AS1. Further experimental detection showed that ATP8B1-AS1 directly activated *MYC* transcription in an m⁶A dependent manner. m⁶A-modified ATP8B1-AS1 bound to m⁶A reader YTHDC1, which further interacted with and recruited the H3K9me2 demethylase KDM3B. Further, we found that ATP8B1-AS1 directly bound to *MYC* promoter region. Thus, only m⁶A-modified ATP8B1-AS1 could recruit KDM3B to *MYC* promoter region, leading to the reducing of H3K9me2 level at *MYC* promoter region and *MYC* transcription activation. Our findings revealed a link between m⁶A-modified lncRNAs and epigenetic modulation and provided novel evidences for the roles of m⁶A modification in epigenetic modulation.⁴⁵

Conclusions

In conclusion, this study identified m⁶A-modified ATP8B1-AS1 as an oncogenic molecular event in HCC. m⁶A modification level of ATP8B1-AS1 is increased and correlated with poor overall survival in HCC. m⁶A-modified ATP8B1-AS1 exerts oncogenic roles in HCC through epigenetically activating *MYC*. Targeting ATP8B1-AS1 or the m⁶A modification of ATP8B1-AS1 represent novel therapeutic strategy for HCC.

Abbreviations

m⁶A, N⁶-methyladenosine; HCC, hepatocellular carcinoma; mRNA, messenger RNA; lncRNA, long noncoding RNA; TCGA, The Cancer Genome Atlas; LIHC, liver hepatocellular carcinoma; FBS, fetal bovine serum; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; NC, negative control; MeRIP, methylated RNA immunoprecipitation; EdU, 5-ethynyl-2'-deoxyuridine; ChIRP, chromatin isolation by RNA purification; ChIP, chromatin immunoprecipitation; AFP, alpha-fetoprotein; SELECT, single-base elongation- and ligation-based qPCR amplification method; GSEA, Gene Set Enrichment Analysis; H3K9me2, histone H3 lysine 9 di-methylation.

Ethics Approval and Consent to Participate

This study was performed following the Helsinki Declaration and with the approval from Youjiang Medical University for Nationalities Institutional Review Board. Written informed consents were acquired from all participants.

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Disclosure

The authors report no conflicts of interest in this work.

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