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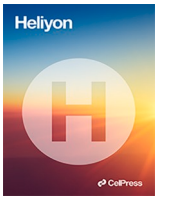

The m6A reader YTHDC1-mediated lncRNA CTBP1-AS2 m6A modification accelerates cholangiocarcinoma progression

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ABSTRACT

Background: Cholangiocarcinoma (CCA) is a serious malignancy originating from the bile ducts and the second most common primary liver cancer. Long non-coding RNA (lncRNA) is a functional lncRNA that plays an important role in human cancers. However, the role and underlying mechanisms of CTBP1-AS2 in CCA remain unknown.

Purpose: In this study, we investigated the functional role and mechanism of long-stranded non-coding RNA (lncRNA) C-terminal binding protein 1 antisense RNA 2 (CTBP1-AS2) in CCA progression.

Result: In the present study, the bioinformatics analysis revealed that YTHDC1 and CTBP1-AS2 were significantly upregulated, and it was confirmed in cholangiocarcinoma tissues from CCA patients. Meanwhile, we demonstrated that knockdown of YTHDC1 or lncRNA CTBP1-AS2 inhibited CCA cell proliferation, migration and invasion, blocked the cell cycle in G2/M phase and promoted apoptosis of CCA cells. In addition, lncRNA CTBP1-AS2-mediated N6-methyladenosine (m6A) methylation levels were significantly elevated in cholangiocarcinoma tissues, whereas knockdown of YTHDC1 resulted in a significant down-regulation of m6A methylation levels by lncRNA CTBP1-AS2.

Conclusion: Our results suggest that YTHDC1 affects cholangiocarcinoma progression by modifying the lncRNA CTBP1-AS2 m6A, and CTBP1-AS2 may be a promising therapeutic target for CCA.

1. Introduction

Cholangiocarcinoma (CCA) is a rare tumor originating from the bile duct epithelium [1]. According to statistics, the incidence of CCA in Western countries is lower than that in China, where the incidence of CCA is three times higher than that in the United States, where 5000 new cases are diagnosed annually [2]. The clinical features of the patients in the early stages of the disease are extremely inconspicuous, resulting in advanced cancer at the time of diagnosis, which is difficult to resolve by surgical resection at this point in time. Prognostic care for cholangiocarcinoma usually consists of nursing care, dietary care, physical activity, drainage care, and regular checkups. Current therapeutic tools, the regulatory role of the genome, and the technology of sensors have opened up new avenues for the diagnosis and treatment of cancer [3,4]. Therefore, it is extremely urgent to reveal the intrinsic mechanisms of CCA progression in order to develop new therapeutic strategies with reference to existing novel approaches to cancer treatment.

In recent years, RNA epigenetics have become a hot topic, and N6-methyladenosine (m6A) transcriptional modifications have

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received increasing attention as one of these forms. m6A is the most abundant internal modification in eukaryotic mRNAs and is dynamic and reversible, regulated by methyltransferases (writers), demethylases (erasers) and effector proteins (readers) [5–8]. There is now growing evidence that m6A modifications are closely associated with the development of a variety of cancers. Methyltransferase 3 (METTL3) and methyltransferase 14 (METTL14) [9], as components of the m 6A methyltransferase complex are dysregulated in hepatocellular carcinoma and function as oncogenes in acute myeloid leukemia [10]. Fat mass and obesity-associated protein (FTO) and ALKB homolog 5 (ALKBH5) were shown to be differentially expressed in renal cell carcinoma as m6A demethylases and could be new candidate biomarkers for the management of patients with renal cell carcinoma [11]. FTO has oncogenic properties in both gastric and bladder cancers and could be used as a diagnostic or prognostic biomarker [12,13].

YTHDC1 as an m6A reader in the regulation of RNA metabolism, has a unique function, it is upregulated in oesophageal cancer and acute myeloid leukemia, and plays an important role in promoting metastasis in triple-negative breast cancer [14–16]. However, cholangiocarcinoma is highly malignant, invasive, and poorly treated, making it the most intractable type of cancer among all types of cancers. However, m6A modification plays an important role in cancer. Meanwhile, relatively few studies have been conducted on m6A modification and cholangiocarcinoma, and its mechanism is not clear. In particular, m6A-modified lncRNAs can induce value-addition, migration, and apoptosis in tumor cells, including pancreatic, ovarian, and hepatocellular carcinomas. A related study has shown that a Y-linked lncRNA, LINC00278, which is down-regulated in male ESCC, and smoking can down-regulate the m6A modification of ILNC00278 translation [17]. Thus, m6A-modified lncRNAs play an important role in cancer. Meanwhile, long-stranded non-coding RNAs (lncRNAs) are transcribed RNA molecules up to 200 nucleotides in length with the ability to interact directly with DNA, RNA and proteins, and have a facilitative or inhibitory role in the regulation of gene expression. In recent years, a variety of lncRNAs have been found to play an important role in the occurrence, development, diagnosis, and prognosis of CCA. For example, lncRNA TM4SF1-AS1 acts as a tumor promoter for CCA by regulating miR-744–3p [18], lncRNA FOXD2-AS1 promotes proliferation, migration, and invasion of CCA by regulating the miR-760/E2F3 axis [19], lncRNA 00261 and lncRNA CCAT2 are overexpressed in cholangiocarcinoma clinical specimens and cell lines expression, suggesting a poor prognosis for bile duct cancer [19,20]. However, the mechanism of action between m6A regulators and dysregulation of lncRNAs in cancer development remains unclear, and few studies have focused on the relationship between m6A modifications and lncRNA-dependent CCA. Therefore, understanding the relationship between m6A modification of lncRNAs and the progression of CCA may help identify potential biomarkers as therapeutic targets.

In this study, we explored the role of YTHDC1, a major m6A 'reader', and lncRNA CTBP1-AS2 in CCA. We found that YTHDC1 and lncRNA CTBP1-AS2 were significantly upregulated in CCA tissues. Knockdown of YTHDC1 or lncRNA CTBP1-AS2 inhibited CCA cell proliferation, migration, and invasion, stalled the cell cycle in the G2/M phase and promoted apoptosis of CCA cells. In addition, the m6A methylation level of lncRNA CTBP1-AS2 was significantly elevated in cholangiocarcinoma tissues, whereas the m6A methylation level of lncRNA CTBP1-AS2 was significantly down-regulated after knockdown of YTHDC1, suggesting that YTHDC1 affects the progression of cholangiocarcinoma by modifying lncRNA CTBP1-AS2 m6A. In conclusion, our study highlights the important roles of YTHDC1 and lncRNA CTBP1-AS2 in CCA, providing new insights into the tumorigenesis of CCA and new therapeutic targets for CCA.

2. Materials and methods

2.1. Differentially expressed (DE) lncRNAs, and m6A-related genes in CCA

We obtained transcriptome sequencing (RNA-seq) information for 9 normal control samples, and 36 cholangiocarcinoma tumor samples with corresponding clinical data using the UCSC Xena database (https://xenabrowser.net/datapages/) updated on December 01, 2021. Then using the limma R package, the sorted lncRNAs expression matrix was used for gene difference analysis ($|\log2FC|>1, p < 0.05$) and these up- or down-regulated genes were shown by plotting volcano and heat maps. pearson correlation analysis was used to identify lncRNAs associated with m6A ($|\text{Pearson R}| > 0.35$, $p < 0.025$). Prediction of lncRNAs with linkage to m6A enzymes using the m6A methylesterase corresponding target gene database m6A2Target (http://m6a2target.canceromics.org/) database.

2.2. Detection of patient tissues

Cholangiocarcinoma cancer tissues and their paired normal tissues (para-cancer) were obtained from patients who were diagnosed with bile duct cancer and underwent surgery at the First Affiliated Hospital of Jilin University in 2022. All patients signed an informed consent form before the use of clinical material. The tissues used in this study were certified by the Ethics Committee of the First Affiliated Hospital of Jilin University. The tissues were used for Immunohistochemical (IHC) assay and For Real-time quantitative PCR (RT-PCR).

2.2.1. IHC assay

Cancer and para-cancer tissues were fixed in 4% paraformaldehyde for 24 h and routinely paraffin-embedded to make 4 μm sections. The section was soaked in 3% H_2O_2 for 15 min, incubated with non-immune goat serum for 1 h at 37 °C and then incubated with anti-YTHDC1 (29441-1-AP, Proteintech, USA) overnight at 4 °C. Then incubated with horseradish enzyme-labeled secondary antibody (29441-1-AP, Proteintech, USA) for 30 min at 37 °C. Subsequently, they were stained with DAB reagent (ZLI-9018, ZhongShan JinQiao, Beijing, China) for 1 min. Histomorphological changes were observed and photographed under a microscope (IX70, Olympus, Japan).

2.2.2. RT-PCR assay

The tissue specimens were ground and tissue (~30 mg) total RNA was extracted using Trizol reagent under liquid nitrogen freezing. Total RNA was then reverse transcribed to cDNA using PrimeScript™ RT Reagent Kit (RR036A, Takara, Japan). Finally, the target genes were subjected to fluorescent quantitative PCR using Power SYBR Green PCR Master Mix (4367659, Thermo, USA). The results were analyzed using $2^{-\triangle\triangle}$Ct quantification method. GAPDH was used as the internal reference and all primers were synthesized by Shanghai Sangon Biotech (Table 1).

2.3. Detection of cell

HuCCT1 cells were purchased from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (C11875500BT, Gibco, USA) with 10% fetal bovine serum (16000–044, Gibco, USA) and 1% penicillin/streptomycin (15140–122, Gibco, USA), and were incubated at 37 °C in a 5% CO_2 incubator and changed every 2–3 days in culture medium.

The siRNAs used in this study were purchased from Shanghai Jereh Bio (Shanghai, China). The siRNA was transiently transfected into HuCCT1 cells using Lipofectamine 2000 transfection reagent (11668–027, Thermo, USA). Cells were inoculated into 24-well plates, Lipofectamine 2000 and siRNA were added to serum-free medium, mixed and incubated for 20 min at room temperature. The siRNA-Lipofectamine 2000 mixture was carefully added to each well and the cells were then incubated at 37 °C in a humidified environment with 5% CO_2. 48 h later RT-PCR was used to detect transfection efficiency and to perform subsequent experiments. After transfection of cells with siRNAs, the siRNAs with the best knockdown efficiency were further identified for subsequent use in cell proliferation, migration and invasion, cell cycle and apoptosis, RT-PCR and Western blot assays. The experiment was divided into four groups: control, si-NC, si-CTBP1-AS2, and si-YTHDC1, and the experimental flow chart is shown in Fig. 3A.

2.3.1. Cell viability and clone formation assays

CCK-8 Proliferation Kit (C0038, Beyotime, China) was used for the assay and the operation was performed according to the instructions. In addition, for clone formation assay, the cells were spread in 6-well plates at a density of 400 cells/well and incubated in the incubator for 14 days. When obvious cell clones were visible to the naked eye, the supernatant was discarded, then 4% paraformaldehyde was added and fixed at room temperature for 10 min, 4% paraformaldehyde was discarded and washed twice with PBS, crystalline violet was stained at room temperature for 10 min, the excess crystalline violet was gently washed off with distilled water, dried at room temperature, observed, and photographed.

2.3.2. Cell migration and invasion assays

Transwell assays were performed to detect cell invasion and migration. For cell migration assays, cells were digested with trypsin, resuspended in a serum-free medium and the cell density was adjusted to 5×10^5 cells/ml. A sterile 24-well plate was taken and 500 μl of complete medium was added to the wells. Transwell chambers of 8 μm pore size were taken with forceps and gently placed in the 24-well plate containing medium. And, 200 μl was pipetted into the upper chamber of the transwell. After 48 h of incubation, the chambers were removed, fixed at room temperature by adding 4% paraformaldehyde for 20 min, and stained with crystal violet staining solution for 20 min at room temperature. The cells in the upper chamber of the transwell were carefully wiped off with a

Table 1
The primer sequences for RT-PCR.

Primers	(5′ to 3’)	
LINC01018	F	TGGATTCACATCTGCTGGGT
	R	TGGCCAACATTTGTCAAGGG
CTBP1-AS2	F	CAAGGGCACTCAAAGGGCTA
	R	CAGGCAGGCAAACACAGAAC
YTHDC1	F	AACTGGTTTCTAAGCCACTGAGC
	R	GGAGGCACTACTTGATAGACGA
FTO	F	ACTTGGCTCCCTTATCTGACC
	R	TGTGCAGTGTGAGAAAGGCTT
CTBP1-AS2-F(MeRIP)	F	GGGACGATGTGGTTGGTG
	R	AAAGCCTTATGTGATGTTTGC
GAPDH	F	TGACAACTTTGGTATCGTGGAAGG
	R	AGGCAGGGATGATGTTCTGGAGAG
CTBP1-AS2 si-1	F	GCUAAAUCAAGUUAAUUAATT
	R	UUAAUUAACUUGAUUUAGCTT
CTBP1-AS2 si-2	F	GGCAAAUAAUACUUGUAUATT
	R	UAUACAAGUAUUAUUUGCCTT
CTBP1-AS2 si-3	F	GGCUAAAUCAAGUUAAUUATT
	R	UAAUUAACUUGAUUUAGCCTT
YTHDC1 si-1	F	GGAAGAUGAAGAAGUAGAATT
	R	UUCUACUUCUUCAUCUUCCTT
YTHDC1 si-2	F	GGAGGAAGAUGAAGAAGUATT
	R	UACUUCUUCAUCUUCCUCCTT
YTHDC1 si-3	F	AGAAGAAGAUGCAGAAGAATT
	R	UUCUUCUGCAUCUUCUUCUTT

cotton swab, and the stained cells were counted under an inverted microscope and photographed. For the detection of cell invasion, the upper chamber of the transwell should be pre-coated with matrix gel (354234, Corning, USA), otherwise, the procedure is the same as for the cell migration assay.

2.3.3. Cell cycle and apoptosis assays

Cells were gently resuspended in PBS, centrifuged and the supernatant discarded. Add 195 μl Annexin V-FITC conjugate and gently resuspend the cells according to the instructions of the Annexin V-FITC/PI Apoptosis Assay Kit (556420, BD, USA), add 5 μl Annexin V-FITC and mix gently, add 10 μl PI staining solution and mix gently, incubate at room temperature. Apoptosis is detected using a flow cytometric analyzer (FACSCalibur, BD, USA).

Flow cytometry for cell cycle detection: After transfection, cells are digested with appropriate amounts of trypsin and collected in flow tubes, centrifuged and the supernatant discarded. cells are gently resuspended in PBS, 4 mL of 70% ethanol pre-chilled at −20 °C is added, mixed well and the cells are fixed overnight at 4 °C in a refrigerator. Centrifuge and discard the supernatant, add PBS, and wash the cells once, centrifuge and discard the supernatant, add PBS containing 50 μg/ml RNase A (ST578, Beyotime, China). Add 20 μl of PI to a final concentration of 50 μg/ml, stain for 30 min protected from light and use a flow cytometric analyzer to detect the cell cycle.

2.3.4. RT-PCR assay

Cells were collected, and lysed using Trizol (9109, TaKaRa, Japan) and total RNA was extracted using Trizol reagent under liquid nitrogen freezing, and the follow-up on methodology 2.2.2. GAPDH was used as the internal reference and all primers were synthesized by Shanghai Sangon Biotech (Table 1).

2.3.5. Western blot

Total protein was extracted from the cells using RIPA lysate (P0013B, Beyotime, China) containing PMSF (ST506, Beyotime, China) and the protein concentration was determined using the BCA kit (20201ES76, Yisheng Biotechnology Co., Ltd., China). The primary antibody incubation was performed with the antibodies in Table 2. HRP-labeled secondary antibody was used for 1 h on the following day. ECL kit was used for membrane incubation. ECL kit (P0018S, Biyotime, China) was used for chemiluminescence development. Using GAPDH as the internal reference, the grey value of each band was analyzed using the gel image analysis software Image J. The ratio of the grey value of the target protein to the internal reference protein band was calculated.

2.4. Methylated RNA immunoprecipitation (MeRIP) assay

MeRIP assays were performed to identify m6 A modifications on individual transcripts using the GenSeq® m6A MeRIP kit (GS-ET-001, Cloud Sequence Bio, China) according to the manufacturer's recommendations. Briefly, total RNA was isolated from pre-treated tissues or cells and randomly split into nucleotide sizes of 100 or less. RNA samples were then immunoprecipitated using magnetic beads pre-coated with 10 μg of anti-m6 A antibody (ab208577, Abcam, UK) or anti-mouse IgG (ab170190, Abcam, UK). N6-methyladenosine 5′-monophosphate sodium salt (6.7 mM) was applied to elute the m6 A-modified RNA fragments. qPCR analysis was then performed to determine the modification of specific genes by m6A.

2.5. Fluorescence in situ hybridization (FISH)

The FISH kit (C10910, Guangzhou RiboBio, China) was used to detect the distribution of lncRNA CTBP1-AS2 expression in the HuCCT1 cell line. Cell crawls were placed in the bottom of 24-well plates, 5×10^4/well, and the supernatant was removed after 24 h of incubation. 1 × PBS was used to wash the cells, and after being fixed in 4% paraformaldehyde, PBS containing 0.5% Triton X-100 (T9284-100 ML, Sigma, USA) was added and left at 4 °C for 5 min. Pre-hybridization solution was closed at 37 °C for 30 min, and the LncRNA CTBP1-AS2 probe was hybridized overnight at 37 °C, cells were washed with hybridization wash at 42 °C protected from light, and crawls were fixed on slides with blocker (containing DAPI) under light-protected conditions. Experimental specimens were observed under laser confocal microscopy detection.

Table 2
Information about proteins.

Protein name	Company	Catalog Number
YTHDC1	Proteintech	29441-1-AP
BAX Antibody	Proteintech	50599-2-Ig
BCL-2 Antibody	Proteintech	26593-1-AP
CCNB1 Antibody	Proteintech	28603-1-AP
P53 Antibody	Proteintech	60283-2-Ig
PTEN Antibody	Proteintech	22034-1-AP
SMAD2 Antibody	Proteintech	12570-1-AP
SMAD3 Antibody	Proteintech	66516-1-Ig
TGF-β1 Antibody	Proteintech	21898-1-AP
GAPDH Antibody	Proteintech	60004-1-Ig

2.6. Statistical analysis

The data obtained from the experiment were expressed as mean ± standard deviation, and all data were processed and statistically analyzed using GraphPad Prism 6. Paired t-tests were used to compare the data between two groups following a normal distribution and a chi-square paired design, and one-way ANOVA was used to compare the data between multiple groups. $P < 0.05$ indicates that the difference is statistically significant.

3. Results

3.1. Identification of DE lncRNAs and m6A-related genes in CCA

The flow of the experiment is shown in Fig. 1A. A total of 869 lncRNAs (Fig. 1 B–C) and 21 m6A-related genes (Table 3) were screened for differential expression in 36 cholangiocarcinoma tumor samples and 9 normal control samples, and these differentially expressed RNAs could well distinguish tumor tissue from normal tissue. We further compared the significantly differentially expressed lncRNAs obtained from the screening with lncRNAs with linkage to m6A enzymes, retaining the intersection part, and obtained a total of 1395 linkage pairs (Supplementary material Table). The two m6A methylation genes FTO and YTHDC1, and the more significantly differentially expressed lncRNA 01018 and lncRNA CTBP1-AS2 associated with them, were selected according to the results shown.

3.2. Verification of bioinformatics analysis

Based on the results of bioinformatics analysis, we further verified the expression of FTO, YTHDC1, lncRNA 01018 and lncRNA CTBP1-AS2 in para-cancer and cholangiocarcinoma tissues using RT-PCR and IHC. The expression levels of FTO were significantly down-regulated, however, the expression levels of YTHDC1 and CTBP1-AS2 were significantly up-regulated (Fig. 2 A-D). Consistent with these results, IHC staining showed elevated YTHDC1 expression in cholangiocarcinoma tissues (Fig. 2 E). To further explore the relationship, we further used the MeRIP assay. After immunoprecipitation of m6A-methylated lncRNA CTBP1-AS2 with m6A antibody followed by quantification by RT-PCR, we found that the level of methylated lncRNA CTBP1-AS2 was significantly higher in

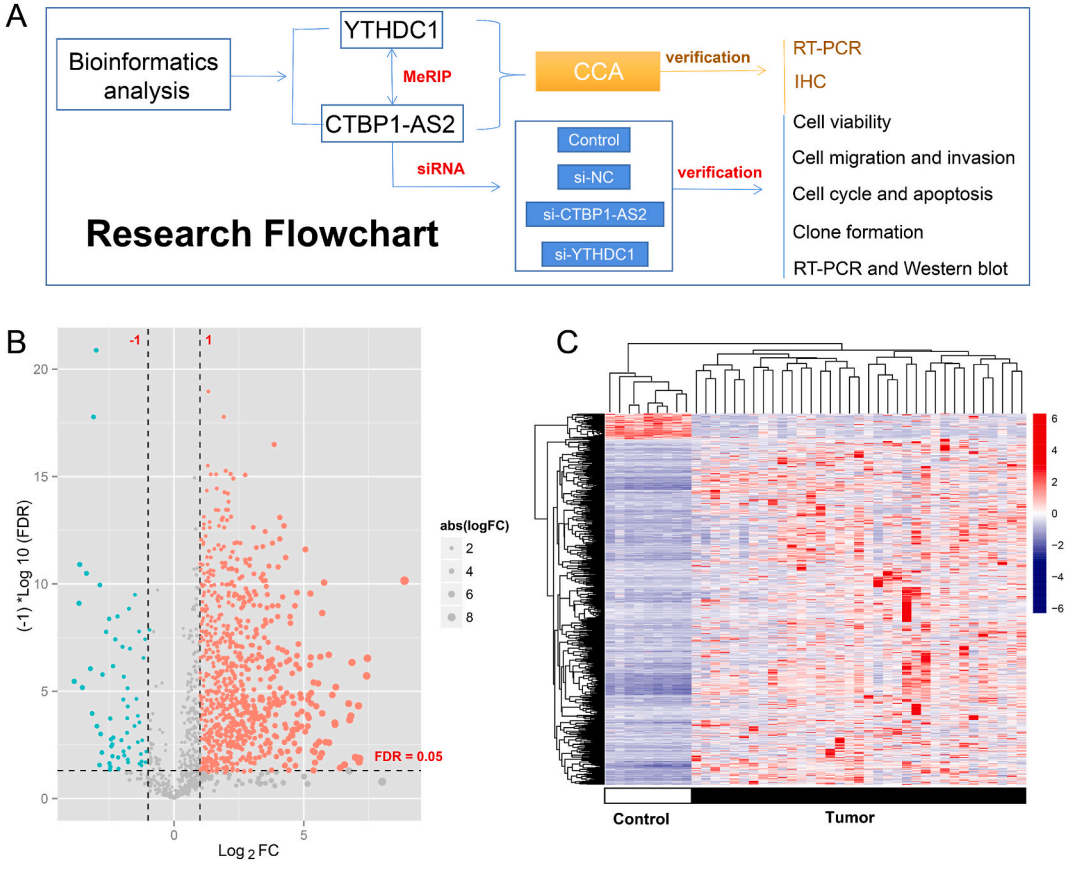

Fig. 1. Identification of DE lncRNAs and m6A-related genes in CCA. (A) Research Flowchart (B) Volcano map of differentially expressed lncRNAs (red dots represent up-regulated genes, green dots represent down-regulated genes). (C) Cluster heat map of the hetero-expressed gene.

Table 3
The significant differential expressed m6A-related genes in the cholangiocarcinoma (CCA).

Symbol	Ensembl_ID	Type	logFC	p-value
FTO	ENSG00000140718.17	m6A	0.781006893	1.76E-16
YTHDC1	ENSG00000083896.11	m6A	0.565687518	9.38E-14
YTHDF1	ENSG00000149658.16	m6A	0.495255095	1.63E-17
VIRMA	ENSG00000164944.10	m6A	0.747802254	4.01E-17
HNRNPA2B1	ENSG00000122566.19	m6A	0.31534188	1.15E-21
IGF2BP2	ENSG00000073792.14	m6A	2.369131672	5.72E-15
HNRNPC	ENSG00000092199.16	m6A	0.407038525	8.42E-19
RBM15B	ENSG00000259956.1	m6A	0.708435087	3.37E-12
RBMX	ENSG00000147274.13	m6A	0.728362159	5.30E-11
METTL3	ENSG00000165819.10	m6A	0.809934547	6.10E-11
YTHDF2	ENSG00000198492.13	m6A	0.359545756	1.18E-08
WTAP	ENSG00000146457.13	m6A	0.524821543	2.11E-08
RBM15	ENSG00000162775.13	m6A	0.656449031	2.89E-08
METTL15	ENSG00000169519.18	m6A	0.559400741	6.49E-08
YTHDC2	ENSG00000047188.14	m6A	0.631996517	1.80E-07
ALKBH5	ENSG00000091542.8	m6A	0.144697554	6.92E-06
YTHDF3	ENSG00000185728.15	m6A	0.268938846	2.13E-05
IGF2BP3	ENSG00000136231.12	m6A	4.692963109	2.63E-05
IGF2BP1	ENSG00000159217.8	m6A	4.56786277	4.75E-05
ZC3H13	ENSG00000123200.15	m6A	0.271413136	1.76E-02
METTL14	ENSG00000145388.13	m6A	0.150535022	2.71E-02

cholangiocarcinoma tissues compared to para-cancer tissues (Fig. 2 F). To observe where the lncRNA CTBP1-AS2 functions in the cell, we used Fluorescence in situ hybridization (FISH) analysis. It was showed that lncRNA CTBP1-AS2 was mainly expressed in the cytoplasm (Fig. 2 G). Together, these results suggest that YTHDC1 and lncRNA CTBP1-AS2 are aberrantly expressed in cholangiocarcinoma and may be involved in the development of CCA.

3.3. Cell transfection efficiency using RT-PCR

However, we further investigated how YTHDC1 and lncRNA CTBP1-AS2 affect the process of CCA. Therefore. we constructed three CTBP1-AS2 interference sequences and three YTHDC1 interference sequences, respectively. RT-PCR results showed that compared with the si-NC group, the expression of the si-CTBP1-AS2(1), si-CTBP1-AS2(2) and si-CTBP1-AS2(3) groups was significantly reduced, with si-CTBP1 -AS2(1) group had lower relative expression and the best silencing effect. Similarly compared with si-NC, the expression of si-YTHDC1(1), si-YTHDC1(2) and si-YTHDC1(3) groups were significantly reduced, with si-YTHDC1(3) group having lower relative expression and the best silencing effect, so si-CTBP1-AS2(1) and si-YTHDC1(3) were utilized for subsequent experiments (Fig. 3A–B). Further RT-PCR results showed that the expression of YTHDC1 was significantly lower in the si-CTBP1-AS2 group compared to the si-NC group, and similarly the expression of CTBP1-AS2 was significantly lower in the si-YTHDC1 group compared to the si-NC group, indicating that YTHDC1 and CTBP1-AS2 can influence each other (Fig. 3C–D).

3.4. Effects of CTBP1-AS2 and YTHDC1 on cell viability, migration, and invasion

After screening the effective siRNAs of CTBP1-AS2 and YTHDC1, we further examined the effects on cell proliferation, migration and invasion after knockdown. The CCK-8 assay showed that the knockdown of CTBP1-AS2 or YTHDC1 resulted in a significant decrease in cell activity at 48 and 72 h (Fig. 4 A). Transwell migration and invasion assays showed that knockdown of CTBP1-AS2 or YTHDC1 resulted in a significant decrease in cell migration and invasion compared to the control group (Fig. 4 B).

3.5. Effects of CTBP1-AS2 and YTHDC1 on cell apoptosis, cycle, and clone formation

The above results suggest that knockdown of CTBP1 -AS2 or YTHDC1 can affect cell proliferation migration and invasion. In order to further verify that the effect on cell growth after knocking down these two genes, we therefore examined cell growth cycle, apoptosis and clone formation. Flow cytometry was used to detect the effects of the knockdown of CTBP1-AS2 or YTHDC1 on cell apoptosis and cycle. The results showed that knockdown of CTBP1-AS2 or YTHDC1 induced apoptosis in cholangiocarcinoma cells compared to controls (Fig. 5A), increasing the number of cells in the G2/M phase and a decrease in the number of cells in the S phase (Fig. 5 B). Next, we examined the clonogenic ability of cholangiocarcinoma cells and found that knockdown of CTBP1-AS2 or YTHDC1 inhibited clone formation compared to controls (Fig. 5 C).

3.6. Effects of CTBP1-AS2 and YTHDC1 on the progression of cholangiocarcinoma

The growth process and growth rate of cancer cells is often accompanied by apoptosis and the two are inversely proportional. The above results detected that knockdown of CTBP1-AS2 or YTHDC1 induced apoptosis in cholangiocarcinoma cells, and we further

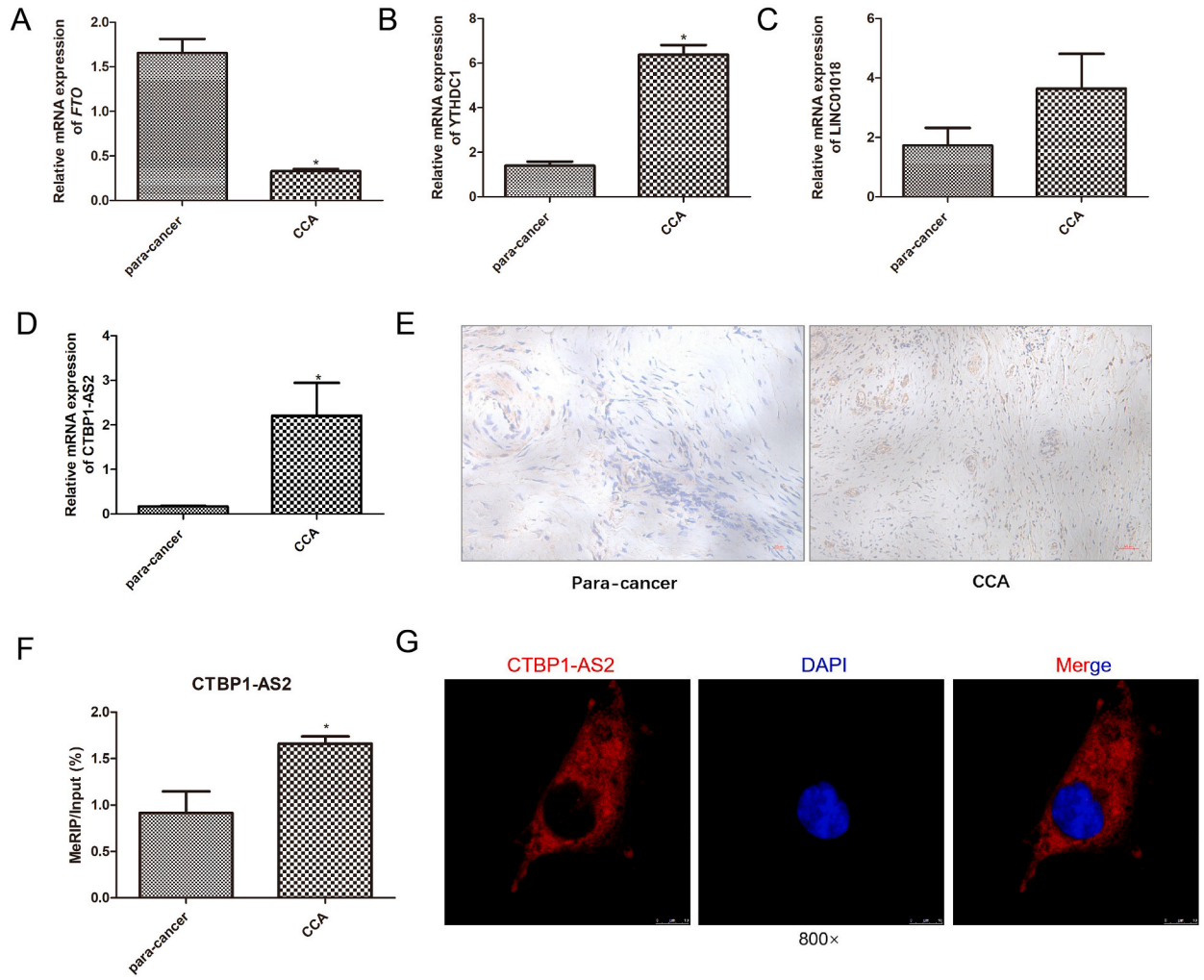

Fig. 2. Verification of bioinformatics analysis. The mRNA expression of *FTO* (A), *YTHDC1* (B), LINC01018 (C), and *CTBP1-AS2* (D) in para-cancer and CCA. (E) MeRIP-qPCR analysis of CTBP1-AS2 in para-cancer and CCA. (F) The expression of YTHDC1 was determined by immunohistochemistry. (G) Representative images and quantification of CTBP1-AS2 mRNA localization by FISH. $^{*}P < 0.05$ *vs.* para-cancer group. Data are shown as Mean ± SD, n = 3.

examined apoptosis-related proteins to elucidate their key role in the progression of cholangiocarcinoma [21–23]. As shown in Fig. 6 A-E, the protein expression of BCL-2 was significantly downregulated after the knockdown of CTBP1-AS2 or YTHDC1 compared to the blank control or si-NC groups (Fig. 6 B). As shown in Fig. 6 C, the protein expression of CCNB1 was significantly up-regulated after the knockdown of YTHDC1 compared to the blank control or si-NC groups, but there was no significant difference in the protein expression of CCNB1 after the knockdown of CTBP1-AS2. As shown in Fig. 6 D, the expression of p53 was significantly up-regulated after the knockdown of YTHDC1 compared to the blank control. However, there was no significant difference in the protein expression of p53 after the knockdown of CTBP1-AS2 compared to the blank control or si-NC groups. The protein expression of BAX was significantly upregulated after the knockdown of CTBP1-AS2 or YTHDC1 compared to the blank control or si-NC group (Fig. 6 E). This suggests that the knockdown of CTBP1-AS2 or YTHDC1 induced apoptosis in bile duct cancer cells.

Furthermore, PTEN is a haploinsufficient tumor suppressor gene, and genetic ablation of PTEN accelerates the progression of several human cancers and can interfere with cancer progression by modulating the TGF-β/Smad signaling pathway [24–28]. Thus, we further tested the related pathway genes of PTEN. As shown in Fig. 6 F–H, the protein expression of PTEN and SMAD2 was significantly down-regulated after knocking down CTBP1-AS2 or YTHDC1 compared to the blank control or si-NC groups, however, there was no significant difference in the expression of SMAD2, and the expression of P53, BAX and CCNB1 was significantly up-regulated. As shown in Fig. 6 I, the protein expression of TGF-β was significantly down-regulated after the knockdown of YTHDC1 compared to the blank control or si-NC groups. This suggests that the knockdown of CTBP1-AS2 or YTHDC1 downregulates the expression of PTEN and its pathway proteins, thereby inhibiting cancer progression.

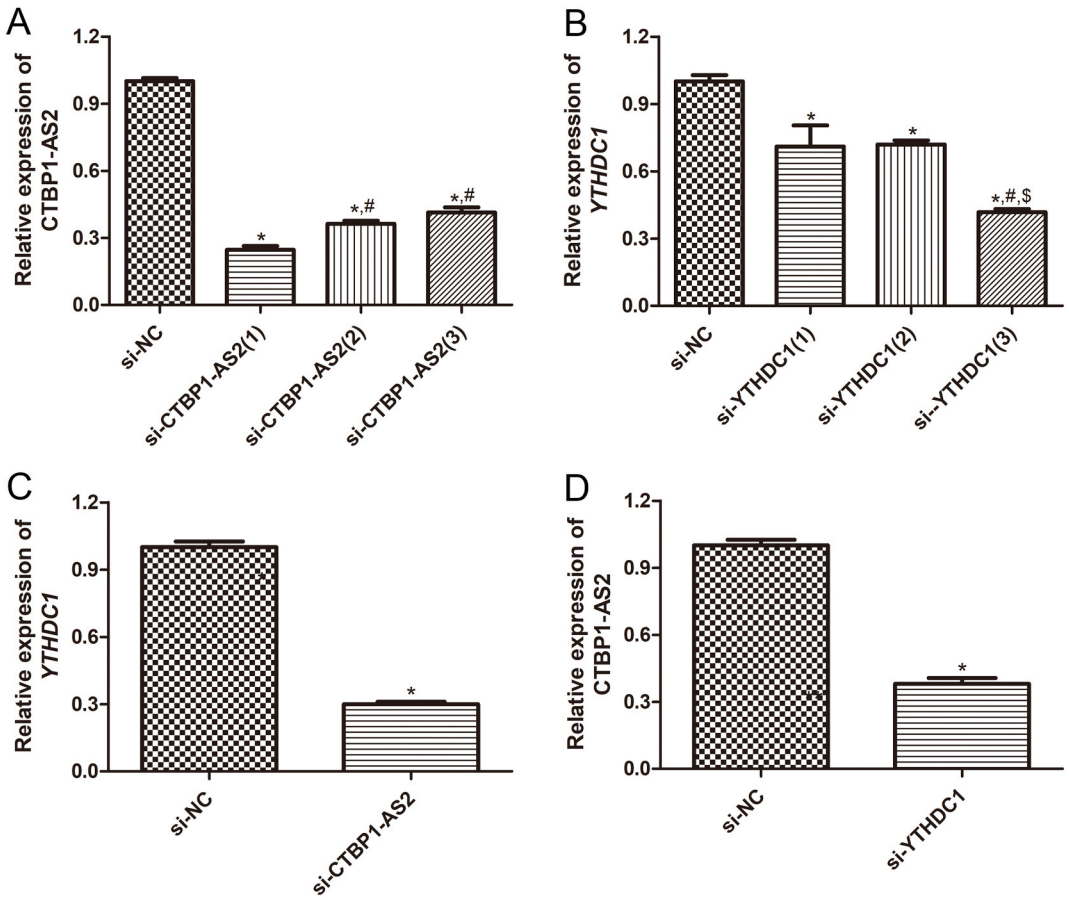

Fig. 3. Cell transfection efficiency using RT-PCR. Transfection efficiencies of CTBP1-AS2 (A) and YTHDC1 (B) in HuCCT1 cell. Relative expression of YTHDC1 (C), CTBP1-AS2 (D) in HuCCT1 cell. $^{*}P < 0.05$ *vs.* siNC group, $^{\#}P < 0.05$ *vs.* si CTBP1-AS2(1) or si YTHDC1(1), $^{\$}P < 0.05$ *vs.* si CTBP1-AS2(2) or si YTHDC1(2). Data are shown as Mean ± SD, n = 3.

3.7. Effects of YTHDC1 on the m6A level of CTBP1-AS2

The above results indicated that knockdown of either CTBP1-AS2 or YTHDC1 could affect the process of CCA, and we further examined the relationship between each other. MeRIP-qPCR examined the effect of YTHDC1 on the m6A level of CTBP1-AS2, and we found that knockdown of CTBP1-AS2 or YTHDC1 resulted in significant downregulation of m6A modification of CTBP1-AS2 in cholangiocarcinoma cells (Fig. 7), suggesting that YTHDC1 mediated the m6A of CTBP1-AS2 methylation.

4. Discussion

Cholangiocarcinoma is a common primary liver malignancy worldwide, and its incidence is increasing year by year, seriously endangering the life and health of patients. Moreover, lncRNAs can serve as important prognostic biomarkers for the development of breast cancer. The present study shows that CTBP1-AS2 is upregulated in CCA patients. Using loss-of-function experiments, we found that CTBP1-AS2 promotes CCA proliferation and metastasis in vitro. Importantly, our study confirms that CTBP1-AS2 upregulation is mediated by m6A modification of YTHDC1.

As we all known, the link between nuclear β-catenin and UF phenotype and β-catenin crosstalk with estrogen and histone deacetylases (HDACs) was explored and it was found that β-catenin nuclear translocation contributes to UF phenotype, and β-catenin signaling is modulated by estradiol and HDAC activity [29]. Moreover, estrogenic compounds as genistein proved to exhibit a neuroprotective effect attributed to its estrogenic, antioxidant, and/or anti-apoptotic properties [30]. Also, it was demonstrated that a novel link between DNA damage and the vitamin D3/VDR axis in UFs. Vitamin D3 suppresses the UF phenotype through orchestrated targeting at multiple molecules in DNA repair pathways, thus offering novel mechanistic insights into the clinical effectiveness of vitamin D3 on UFs [31,32]. Therefore, looking for modifications in genes may be able to inhibit tumor growth through an anti-apoptotic form. In general, epigenetic regulation refers to diverse and reversible chemical modifications of DNA and histones. In addition to DNA and histones, intracellular RNAs (mRNA, lncRNA, snRNA, etc.) also have different types of post-transcriptional modifications, the most common of which is N6-adenosyl methylation (m6A) [33,34]. There is growing evidence that m6A

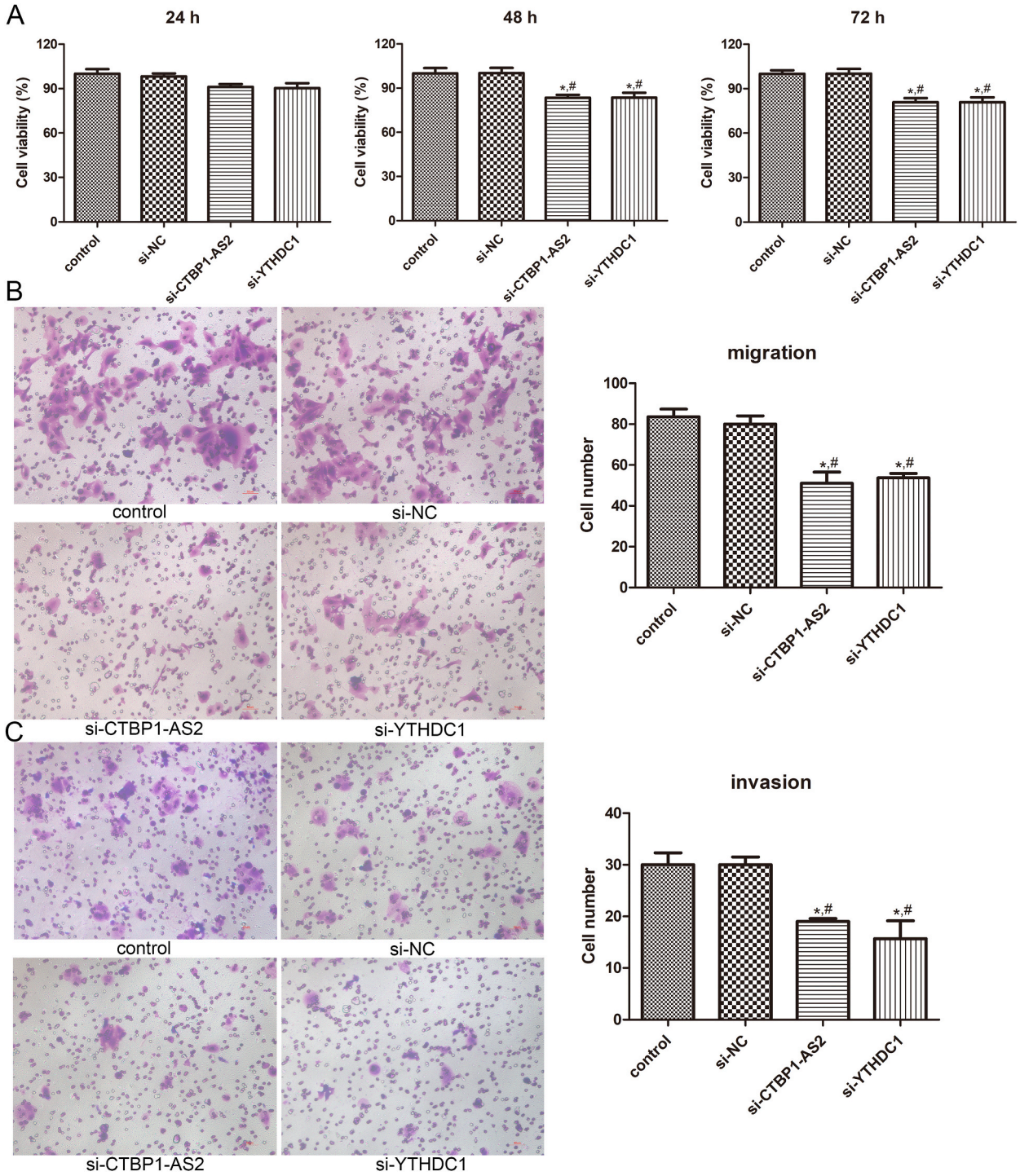

Fig. 4. Effects of CTBP1-AS2 and YTHDC1 on cell viability, migration, and invasion. (A) The cell viability was analyzed by CCK8 assay in HuCCT1 cells. (B–C) The cell migration (B) and invasion (C) were evaluated by transwell assay. $^{*}P < 0.05$ *vs.* control group, $^{\#}P < 0.05$ vs. siNC group. Data are shown as Mean ± SD, n = 3.

modifications play important biological functions in mammals. Long-stranded non-coding RNA (lncRNA) is a type of non-coding RNA that is more than 200 nucleotides in length and usually does not encode proteins and peptides. It has greater spatial and temporal specificity and lower interspecies conservation than mRNA. lncRNAs can be involved in regulating physiological processes such as genomic imprinting, pluripotent differentiation of stem cells, embryonic development, and cardiac development. m6A modifications

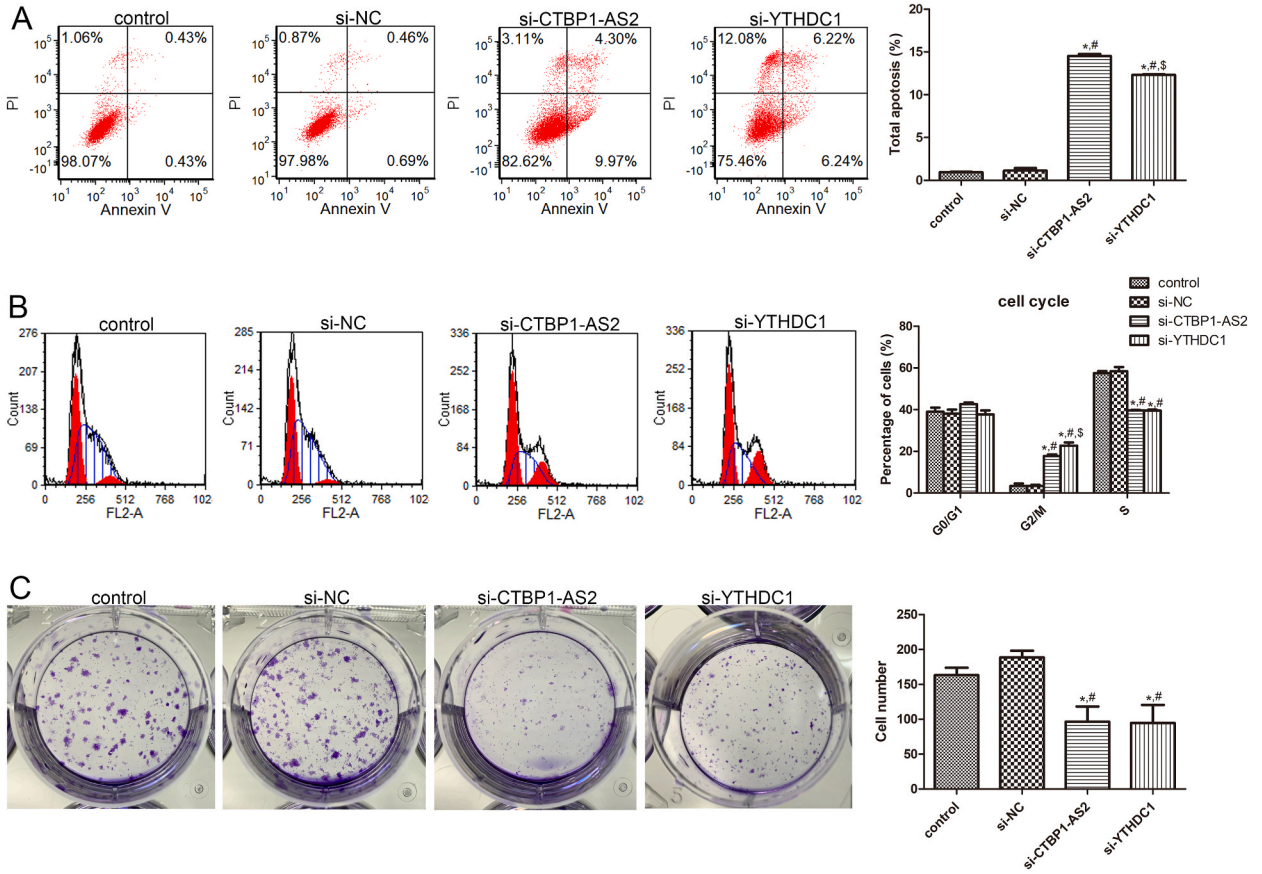

Fig. 5. Effects of CTBP1-AS2 and YTHDC1 on cell apoptosis, cycle, and clone formation. (A) Flow cytometry analysis shows the apoptotic rate; (B) Flow cytometry verified the cell cycle and statistics; (C) Colony formation assay and statistics. $^{*}P < 0.05$ *vs.* control group, $^{\#}P < 0.05$ *vs.* siNC group, $^{\$}P < 0.05$ vs. si CTBP1-AS2 group. Data are shown as Mean ± SD, n = 3.

affect the function of lncRNAs through multiple regulatory mechanisms. On the one hand, m6A modification acts on the RNA-DNA triple helix structure to regulate the relationship between lncRNAs and specific DNA loci [35–37]. On the other hand, m6A modifications provide binding sites for methylated readers (readers) or regulate the structure of local RNAs, thereby inducing the binding of RNA-binding proteins (RBPs) and regulating the function of lncRNAs [38,39]. This study screened two m6A methylation genes, FTO and YTHDC1, and two lncRNAs (CTBP1-AS2 and LINC01018) with significant differential expression for basic experimental validation by bioinformatic functional analysis. CTBP1-AS2 was significantly up-regulated, while we found that the level of methylated lncRNA CTBP1-AS2 was significantly elevated in cholangiocarcinoma tissues, which was consistent with the results observed by IHC staining. FISH analysis also showed that lncRNA CTBP-AS2 was predominantly expressed in the cytoplasm. This part of the experiment suggests that YTHDC1 and lncRNA CTBP1-AS2 may be involved in the development of CCA.

We further validated the important role of YTHDC1 and lncRNA CTBP1-AS2 in the development of CCA by silencing the YTHDC1 and lncRNA CTBP1-AS2 genes and found that cell proliferation, migration and invasion were inhibited after silencing, while the cell cycle became longer and apoptosis was promoted, thus suggesting that YTHDC1 and lncRNA CTBP1-AS2 could accelerate the development of bile duct cancer. To further confirm whether YTHDC1 and lncRNA CTBP1-AS2 promote the development of cholangiocarcinoma, we examined the signature apoptotic protein of the cancer development process, Bcl-2, an apoptosis inhibitory gene, and Bax, which not only antagonizes the inhibitory effect of Bcl-2, but also promotes apoptosis. In this study, we examined apoptosis-related proteins, including BCL-2, PTEN, P53 and BAX, and found that silencing YTHDC1 and lncRNA CTBP1-AS2 significantly increased the levels of pro-apoptotic proteins. Meanwhile, the study reported that circPTEN1, a circular RNA produced by PTEN, could inhibit cancer progression by suppressing the TGF-β/Smad signaling pathway. We also examined PTEN-related pathway proteins, including SMAD2, SMAD3, and TGF-β1, and found that silencing YTHDC1 and lncRNA CTBP1-AS2 significantly reduced the protein levels of PTEN, Sand MAD2. This part of the experiment further suggests that YTHDC1 and lncRNA CTBP1-AS2 can promote apoptosis and decelerate cancer progression.

It was shown that YTHDF and YTHDC family members are generally considered to be readers of m6A methylation and can specifically recognize and bind m6A methylation sites and induce their corresponding functions [40–42]. To further explore the relationship, we used MeRIP-qPCR to detect the effect of YTHDC1 on the m6A level of CTBP1-AS2. We found that knockdown of

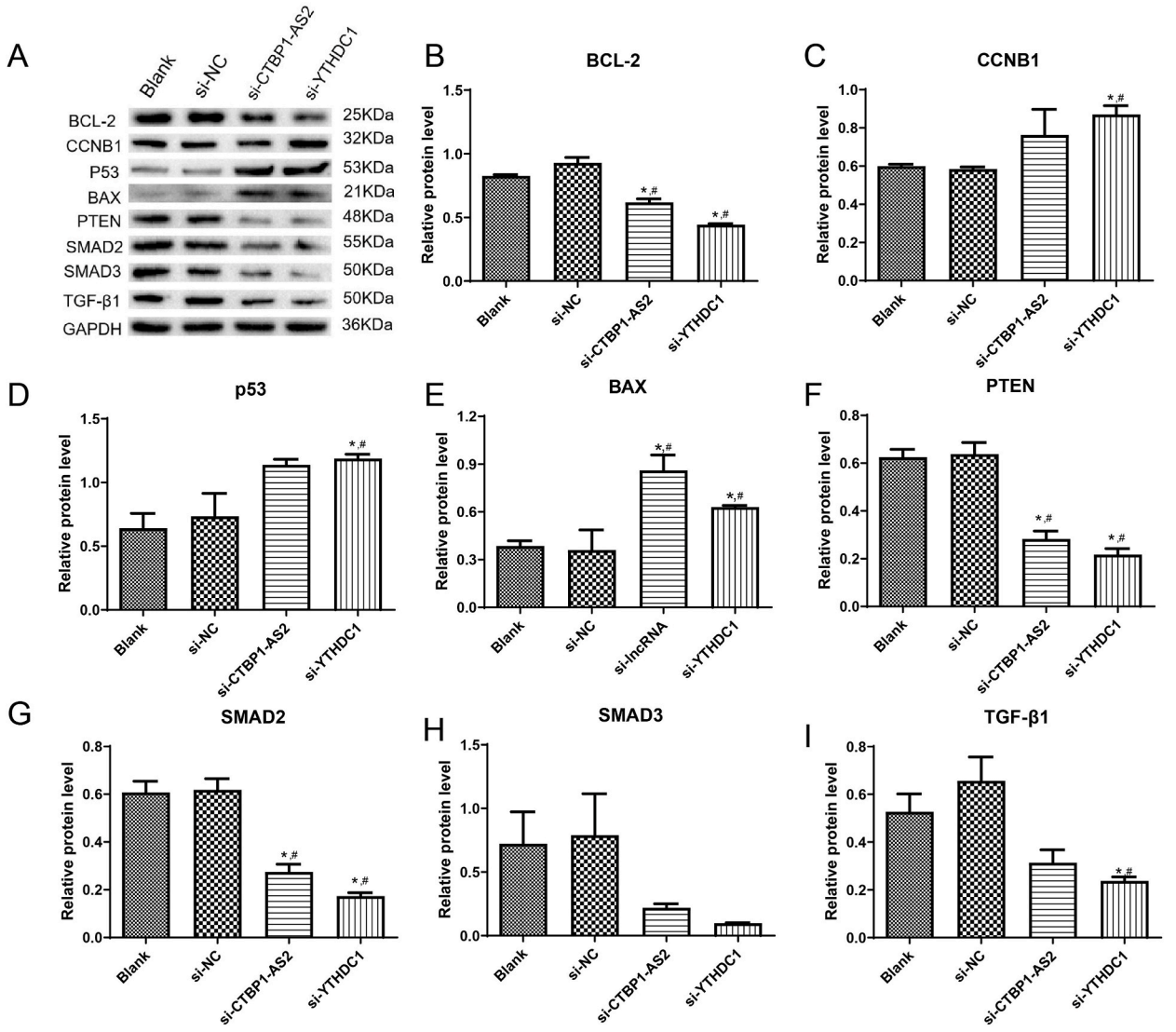

Fig. 6. Effects of CTBP1-AS2 and YTHDC1 on the progression of cholangiocarcinoma. (A–I) Western blot detected the expressions of levels of BCL-2, CCNB1, P53, BAX, PTEN, SMAD2, SMAD3 and TGF-β1 in HuCCT1 cell transfected with si-CTBP1-AS2 and si-YTHDC1. $^{*}P < 0.05$ *vs.* control group, $^{\#}P < 0.05$ vs. siNC group. Data are shown as Mean ± SD, n = 3.

CTBP1-AS2 or YTHDC1 resulted in significant down-regulation of m6A modification of CTBP1-AS2 in cholangiocarcinoma cells, suggesting that YTHDC1 mediates the methylation of CTBP1-AS2 m6A.YTHDC1-mediated attenuation of lncRNA CTBP1-AS2 m6A modification affects the progression of bile duct carcinoma by promoting apoptosis of cholangiocarcinoma cells. However, the limitation of the study is that the role of the currently explored mechanism in other types of cancers is unknown, and therefore, we will subsequently validate it in other types of cancers.

5. Conclusion

Taken together, this study reveals that YTHDC1 and the lncRNA CTBP1-AS2 can affect apoptosis and accelerate cancer progression, providing a promising target for cholangiocarcinoma therapy, in part through YTHDC1-mediated m6A methylation of CTBP1-AS2. Therefore, this research paper is of great importance for the treatment of bile duct cancer. Further studies on m6A methylated lncRNAs will support us to better understand their roles in bile duct diseases or other diseases, especially in tumors. m6A methylation of CTBP1-AS2 mediated by YTHDC1 could provide new research ideas for the treatment of cholangiocarcinoma's in the clinic.

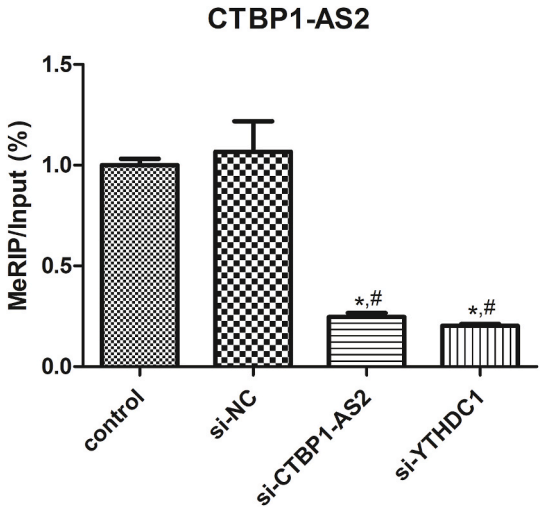

Fig. 7. Effects of YTHDC1 on the m6A level of CTBP1-AS2. MeRIP-qPCR analysis of CTBP1-AS2 in HuCCT1 cell transfected with si-CTBP1-AS2 and si-YTHDC1. $^{*}P < 0.05$ *vs.* control group, $^{\#}P < 0.05$ vs. siNC group. Data are shown as Mean ± SD, n = 3.

Author contribution statement

Yahui Liu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data. Jin Zhe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Ethics approval and consent to participate

All research procedures involving human participants were by the ethical standards of the Research Ethics Committee of the First Hospital of Jilin University (22K101-001) and the 1964 Helsinki Declaration and its later amendments. All written informed consent to participate in the study was obtained from patients with cholangiocarcinoma cancer from whom samples were collected.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19816.

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