lncRNA FLVCR1-AS1 regulates cell proliferation, migration and invasion by sponging miR-485-5p in human cholangiocarcinoma

WENQING BAO, FENG CAO, SHUBIN NI, JING YANG, HAIDONG LI, ZHENXI SU and BIN ZHAO

Department of General Surgery, Shanghai Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai 200135, P.R. China

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Abstract. Long non-coding RNA (lncRNA) FLVCR1 antisense RNA 1 (FLVCR1-AS1) serves a crucial role in many types of cancer; however, to the best of our knowledge, the biological effect of FLVCR1-AS1 in cholangiocarcinoma (CCA) remains unclear. The present study aimed to elucidate the involvement of FLVCR1-AS1 in the regulation of human CCA cell growth, migration and invasion, as well as the mechanisms underlying its effect. The expression levels of FLVCR1-AS1 in CCA tumor tissues, adjacent normal tissues, CCA cell lines and a cholangiocyte cell line were determined by reverse transcription-quantitative polymerase chain reaction. A significantly higher expression level of FLVCR1-AS1 was identified in CCA tumor tissues and the CCA cell lines HuCCT1 and CCLP1 compared with the normal controls. Short hairpin RNA targeting FLVCR1-AS1 (shFLVCR1-AS1) and a control plasmid (shNC) were transfected into CCA cell lines. Cell proliferation, colony formation, migration and invasion of CCA cells transfected with shFLVCR1-AS1 were significantly suppressed compared with the shNC groups. The expression levels of migration and invasion-associated proteins, including Twist, matrix metalloproteinase (MMP)-2 and MMP-9, were also significantly suppressed by shFLVCR1-AS1-treatment. Furthermore, FLVCR1-AS1 knockdown inhibited tumor growth in a xenograft model. Mechanistically, FLVCR1-AS1 was demonstrated to sponge microRNA-485-5p (miR-485-5p) in human CCA. The expression of miR-458-5p was significantly decreased in CCA tissue compared with normal tissue, and Pearson's correlation analysis revealed that FLVCR1-AS1 expression was negatively correlated with miR-485-5p expression in CCA tissues. These results suggested that lncRNA FLVCR1-AS1 may be used as a novel therapeutic target and a potential diagnostic marker for CCA.

Correspondence to: Dr Bin Zhao, Department of General Surgery, Shanghai Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 358 Datong Road, Shanghai 200135, P.R. China
E-mail: zhaobin2016033@126.com

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Introduction

Cholangiocarcinoma (CCA) is a malignant tumor of the extrahepatic bile duct, extending from the hilar area to the lower end of the common bile duct (1). The etiology of CCA may be associated with cholelithiasis, primary sclerosing cholangitis and other types of disease (2). Surgical treatment, radiotherapy and chemotherapy can be used clinically; however, the prognosis is poor (3,4). Consequently, it is crucial to investigate efficient therapies and identify sensitive biomarkers for early diagnosis of CCA. Molecular-targeted therapy has exhibited significant effects against cell proliferation, recurrence and metastasis in CCA, with few adverse effects (5). Therefore, it is important to understand the molecular mechanisms of carcinogenesis and progression in CCA, which may contribute to identifying novel diagnostic markers and sensitive therapeutic targets.

Long non-coding RNAs (lncRNAs) are a class of RNA molecules that are >200 nucleotides and exhibit no protein coding function (6). Recent studies have demonstrated that lncRNAs are closely associated with the development of diverse human diseases, including cancer (7,8). lncRNAs are involved in the regulation of gene expression at the epigenetic, transcriptional and post-transcriptional levels and affect the occurrence, development and invasion of tumors (9,10). Investigations regarding the association between lncRNAs and digestive system tumors may provide a new strategy for the prevention, diagnosis and treatment of these tumors (11,12). Recently, the biological roles and mechanisms of several lncRNAs, including AFAP1 antisense RNA 1 (AS1), ASAP1 intronic transcript 1, colon cancer associated transcript 1, nuclear enriched abundant transcript 1 and sprouty4-intron transcript 1, have been reported to be involved in CCA occurrence and metastasis (13-17). A previous study demonstrated that the expression of lncRNA FLVCR1-AS1 is significantly increased in hepatocellular carcinoma. Additionally, it was identified to promote cancer cell proliferation, migration and invasion by sponging microRNA (miR)-513c in hepatocellular carcinoma (18). However, to the best of our knowledge, the role and function of FLVCR1-AS1 in CCA remains unclear.

The present study identified an increased expression level of FLVCR1-AS1 in CCA tumor tissues and cell lines.

The biological effect of FLVCR1-AS1 on cell proliferation, migration and invasion was investigated. It was revealed that downregulation of FLVCR1-AS1 inhibited these processes. Furthermore, bioinformatics analysis and luciferase reporter assay demonstrated that FLVCR1-AS1 serves an oncogenic role in CCA by sponging miR-485-5p.

Materials and methods

Patients and tissue samples. In total, 22 paired CCA and normal tissue samples were collected from patients (age range, 42-77 years; mean age, 59 years; 13 males and 9 females) who underwent surgical treatment at Shanghai Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) between January 2010 and December 2016. The clinical characteristics of patients with CCA are presented in Table I; the Tumor-Node-Metastasis (TNM) staging system was used as previously described (19). Tissue samples were snap frozen in liquid nitrogen immediately after surgical resection and stored at -80˚C. Patients who received radiotherapy and/or immunotherapy before or following surgery were excluded from the study. Samples were collected after written informed consent was obtained from all patients. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Shanghai Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China).

Cell culture and CCA cell transfection. Human cell lines, including the noncancerous cholangiocyte cell line HIBEC and the CCA cell lines RBE, CCLP1, HuCCT1 and HCCC-9810 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and grown in humidified 5% CO_2 at 37˚C. Short hairpin RNA (shRNA) targeting FLVCR1-AS1 (shFLVCR1-AS1) and the relative control shRNA (shNC) were obtained from Shanghai Genepharma Co., Ltd., (Shanghai, China). The sequence of shFLVCR1-AS1 was 5'-GGTAAG CAGTGGCTCCTCTAA-3' and the sequence of shNC was 5'-AATTCTCCGAACGTGTCACGT-3'. The transfection was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in $5x10^6$ cells at a final concentration of 50 nM, according to the manufacturer's protocol. After transfection for 24 h, expression of FLVCR1-AS1 was validated by reverse transcription-quantitative PCR (RT-qPCR).

RT-qPCR. Total RNA was extracted from tissues and cells using TRIzol® reagent (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Following quantification using Nanodrop 2000 (Thermo Fisher Scientific, Inc.), the extracted total RNA was reverse-transcribed using Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR with SYBR® Green RT-PCR Master mix (Takara Biotechnology, Co., Ltd., Dalian, China) was performed on an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer specific sequences (FLVCR1-AS1, forward, 5'-GTG GCTCTCTCGTTCCC-3' and reverse, 5'-CCGTCCTTCGGT AGTGTC-3'; miR-485-5p, forward, 5'-AGAGGCTGGCCG TGAT-3' and reverse, 5'-ATGTGTTGCTGTGTTTGTCG-3') were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The PCR conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 40 sec and extension at 72˚C for 10 sec. Universal miRNA RT-qPCR primer, 5'-AACGAG ACGACGACAGAC-3'; 5'-GCAAATTCGTGAAGCGTTCCA TA-3' for U6 was used as an endogenous control to normalize miR-485-5p expression level. GAPDH (forward, 5'-TCCTCT GACTTCAACAGCGACAC-3' and reverse, 5'-CACCCT GTTGCTGTAGCCAAATTC-3') was used as an endogenous control to normalize lncRNA FLVCR1-AS1 expression level. The relative expression level was calculated using the $2^{-\Delta\Delta Cq}$ method (20). All the experiments were performed in triplicate.

Cell proliferation (MTT) assay. CCA cells transfected either with shNC or shFLVCR1-AS1 were seeded into 96 well plates at 5,000 cells/well. At 12, 24, 48 and 72 h after transfection, the medium was removed by suction and replaced with serum-free RPMI-1640 medium containing 1 mg/ml MTT. The cells were then incubated at 37˚C for 4 h. Following removal of the MTT solution, the formazan precipitate was dissolved with 100 μl DMSO and the absorbance was measured at 570 and 600 nm in a microplate reader. shNC cells were used as the normal controls.

Colony formation assay. CCA cells were trypsinized, counted and seeded into 12-well plates at 100 cells/well. The medium was replaced every 3 days during colony growth. Following incubation in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 12 days, the colonies were fixed with methanol for 20 min at room temperature and stained with 0.2% crystal violet for 15 min (Sigma-Aldrich; Merck KGaA) at room temperature. The colonies were counted under an inverted microscope (Nikon Corporation, Tokyo, Japan) at magnification, x100.

In vivo xenograft experiments. All animal experiments were performed in accordance with institutional and international animal regulations. The animal protocols were approved by the Animal Care and Use Committee at Shanghai Seventh People's Hospital Affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China). Male 6-week old BALB/c nude mice (N=8) were purchased from Beijing HFK Bioscience Co. Ltd. (Beijing, China). All mice were kept in pathogen-free conditions in 26-28˚C and 50% humidity on a 12 h light/dark cycle. Mice had *ad libitum* access to food and water. For tumor propagation analysis, HuCCT1 cells, transfected either with shNC or shFLVCR1-AS1, were subcutaneously injected into the flanks of BALB/c nude mice at $1x10^6$ cells/mouse. The length and width of tumors were measured using a caliper every 7 days for a period of 28 days. All mice were euthanized at day 28 post-injection, and the tumor nodules of the mice were removed and weighed. The tumor volume was calculated using the following formula: Tumor volume (mm^3)=length (mm) x width $(mm)^2/2$.

Table I. Clinical characteristics of patients with cholangiocarcinoma.

Characteristic	Patients (n=22)
Sex	
Male	13
Female	9
Age, years	
>60	12
≤60	10
Tumor size, cm	
>3	13
≤3	9
Lymph node metastasis	
No	15
Yes	7
TNM stage	
I/II	14
III/IV	8

TNM, Tumor-Node-Metastasis (19).

Wound-healing assay. A wound-healing assay was performed to detect cell migration. In brief, 48 h after transfection, CCA cells were cultured in 6-well plates ($5x10^4$ cells/well). The monolayer of cells was scratched using a sterile plastic micropipette tip and cells were cultured under standard conditions for 24 h. Following several washes, recovery of the wound was observed and images were obtained using a light microscope (magnification, x100; Olympus Corporation, Tokyo, Japan).

Cell invasion assay. After transfection for 48 h, CCA cells ($1x10^5$) transfected with shNC or shFLVCR1-AS1 were seeded into the upper chamber of Matrigel-coated inserts with serum-free medium. RPMI-1640 medium with 10% FBS was added to the lower chamber as a chemoattractant. The cells were allowed to invade for 48 h at 37˚C with 5% CO_2. Migrating cells were fixed in 70% ethanol for 30 min and stained with 0.1% crystal violet for 10 min at 25˚C. The number of cells that migrated to the lower side were counted in five randomly selected fields under an inverted microscope (Olympus Corporation) at x100 magnification.

Luciferase reporter assay. Wild-type FLVCR1-AS1 (5'-AUACACGGCCCUC-3') and mutant FLVCR1-AS1 (5'-AUACUGCCGCCUC-3') sequences were cloned into a pmirGLO reporter vector (Promega Corporation, Madison, WI, USA). HuCCT1 cells were co-transfected with NC mimic (5'-GCAUGAUGGUCAUAGGUCC-3') or miR-485-5p mimic (5'-AGAGGCUGGCCGUGAUGAAUUC-3'), and wild-type FLVCR1-AS1 or mutant FLVCR1-AS1 (1 μg per well) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Relative luciferase activity was measured on a dual-luciferase reporter assay system (Promega Corporation) at 48 h post-transfection. Data are presented as the ratio of *Renilla* luciferase activity to firefly luciferase activity.

Western blot analysis. CCA cells were lysed using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and sonicated. Lysates containing soluble proteins were collected and stored at 80˚C. Protein concentration was determined using a Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total protein lysates (10 μl per lane) were resolved on 10% SDS-PAGE gels and transferred to polyvinyldifluoride membranes (EMD Millipore, Billerica, MA, USA). Following blocking in TBS containing 0.1% Tween-20 (TBS-T) with 5% non-fat dry milk for 30 min at room temperature, membranes were washed four times in TBS-T and incubated with primary antibodies overnight at 4˚C. All primary antibodies were obtained from Abcam (Cambridge, MA, USA) and used at the following dilutions: Anti-Twist (1:500; catalog no. ab49254), anti-matrix metalloproteinase (MMP)-2 (1:500; catalog no. ab92536), anti-MMP-9 (1:500; ab38898), anti-β-actin (1:1,000; catalog no. ab8226). Following extensive washing, membranes were incubated with horseradish peroxidase-linked goat polyclonal anti-rabbit IgG secondary antibodies at a dilution of 1:2,000 for 1 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescence using an ECL kit (Pierce; Thermo Fisher Scientific, Inc.) and exposure to radiography film. GAPDH served as the loading control. Western blots were quantified by densitometry with Labworks Software (version 4.0; UVP BioImaging Systems, Upland, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Two-tailed Student's t-test was applied to compare the differences between two groups and one-way analysis of variance followed by Dunnett's multiple comparison test was used to compare the differences among three independent groups. Correlation between FLVCR1-AS1 and miR-485-5p expression in CCA tissues was identified using Pearson's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference. Each experiment was repeated in triplicate.

Results

Expression of FLVCR1-AS1 in tissues from patients with CCA and cells. To investigate the role of FLVCR1-AS1 in CCA, the expression of FLVCR1-AS1 in 22 CCA and normal tissue samples was analyzed by RT-qPCR. The clinical characteristics of patients with CCA are presented in Table I. It was identified that the expression level of FLVCR1-AS1 was significantly increased in CCA tissues compared with normal tissues ($P<0.001$; Fig. 1A). In addition, RT-qPCR demonstrated that the expression of FLVCR1-AS1 was higher in CCA cell lines (RBE, HCCC-9810, HuCCT1 and CCLP1) compared with the HIBEC noncancerous cholangiocyte cell line ($P<0.01$; Fig. 1B). These results indicated that FLVCR1-AS1 may act as an oncogene in CCA. HuCCT1 and CCLP1 cells exhibited the highest expression of FLVCR1-AS1 and were used for subsequent experiments.

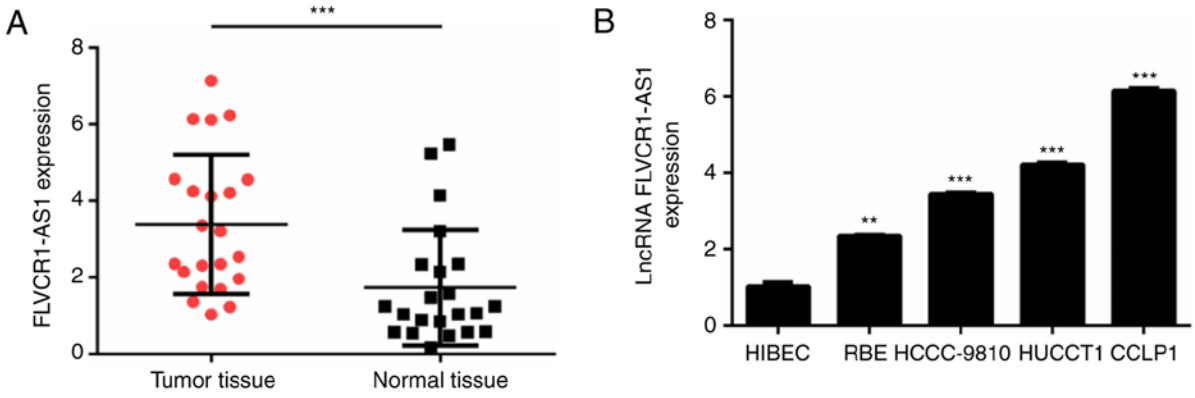

Figure 1. Expression of FLVCR1-AS1 in CCA tissues samples cell lines. (A) The expression level of FLVCR1-AS1 in CCA tissue samples was measured by RT-qPCR (n=22). ***P<0.001. (B) RT-qPCR analysis was performed to determine the expression of FLVCR1-AS1 in the noncancerous cholangiocyte cell line HIBEC and the CCA cell lines RBE, CCLP1, HuCCT1 and HCCC-9810. Data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. HIBEC. CCA, cholangiocarcinoma; FLVCR1-AS1, FLVCR1 antisense RNA 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; lncRNA, long non-coding RNA.

Downregulation of FLVCR1-AS1 inhibits cell proliferation in vitro and in vivo. To investigate whether FLVCR1-AS1 influences cell viability of HuCCT1 and CCLP1 cells, FLVCR1-AS1 was silenced by transfection with shFLVCR1-AS1. The transfection efficiency in HuCCT1 and CCLP1 cells was determined by measuring the expression level of FLVCR1-AS1 using RT-qPCR (Fig. 2A). MTT and colony formation assays were performed to detect cell proliferation and the results revealed that silencing of FLVCR1-AS1 significantly suppressed cell viability and proliferation of HuCCT1 and CCLP1 cells compared with the respective shNC groups (P<0.01; Fig. 2B and C).

The biological role of FLVCR1-AS1 on CCA tumor growth was evaluated in a xenograft mouse model. HuCCT1 cells, transfected with shNC or shFLVCR1-AS1, were implanted subcutaneously into mice. Subsequently, tumor growth was measured every 7 days for a period of 28 days. FLVCR1-AS1-knockdown significantly delayed tumor growth *in vivo* (Fig. 2D). At 4 weeks post-implantation, mice were sacrificed and tumors were harvested and weighed. Silencing of FLVCR1-AS1 significantly decreased the tumor weight (Fig. 2D).

FLVCR1-AS1 suppresses cell migration and invasion. To identify the effect of FLVCR1-AS1 on cell migration and invasion, wound-healing and Transwell assays were performed. As presented in Fig. 3A, the migration ability of cells transfected with shFLVCR1-AS1 was significantly suppressed compared with the shNC groups (P<0.01). The invasion of HuCCT1 and CCLP1 cells transfected with shFLVCR1-AS1 was also significantly reduced compared with the shNC groups (P<0.01; Fig. 3B). Expression levels of migration and invasion-associated proteins, including Twist, MMP-2 and MMP-9, were determined by western blot analysis. The expression levels of Twist, MMP-2 and MMP-9 in the shFLVCR1-AS1 groups were significantly decreased compared with the shNC groups (P<0.01; Fig. 3C).

FLVCR1-AS1 interacts with miR-485-5p in HuCCT1 and CCLP1 cells. To investigate the molecular mechanism by which FLVCR1-AS1 promotes CCA cell proliferation, migration and invasion, target miRNAs of FLVCR1-AS1 were predicted with miRanda. miR-485-5p, which is commonly reported to be involved in many types of human tumor (21,22), was selected as a candidate target of FLVCR1-AS1 (Fig. 4A). Furthermore, luciferase reporter vectors carrying either the predicted miR-485-5p binding site (wild-type FLVCR1-AS1) or its corresponding mutant fragment (mutant FLVCR1-AS1) were constructed. As presented in Fig. 4B, co-transfection of wild-type FLVCR1-AS1 and miR-485-5p mimic significantly reduced the luciferase activity, while co-transfection of mutant FLVCR1-AS1 and miR-485-5p mimic did not affect luciferase activity. The present study further evaluated the expression of miR-485-5p in HuCCT1 and CCLP1 cells transfected with shFLVCR1-AS1 or shNC. As presented in Fig. 4C, the expression of miR-485-5p was significantly increased in the shFLVCR1-AS1 groups compared with the shNC groups (P<0.001). Furthermore, the expression of miR-485-5p in CCA samples and normal tissues was determined by RT-qPCR. A significant decrease was observed in CCA tumor samples compared with the normal tissue samples (P<0.001; Fig. 4D). Pearson's correlation analysis revealed that FLVCR1-AS1 expression was negatively correlated with miR-485-5p expression in CCA tissues (P<0.001; Fig. 4E). These data indicated that FLVCR1-AS1 may interact with miR-485-5p in CCA cells.

Discussion

A number of studies have reported that lncRNAs serve a crucial role in the occurrence, development and prognosis of malignant neoplasms, including CCA (12,23). The present study determined the expression of FLVCR1-AS1 in CCA tissues and cell lines, and examined the effect of FLVCR1-AS1 on CCA cell growth, migration and invasion. In addition, the mechanism by which FLVCR1-AS1 functions as an oncogene in CCA was evaluated.

FLVCR1-AS1 is understood to exhibit tumor-promoting activity in hepatocellular carcinoma (24). Initially, the present study identified that the expression of FLVCR1-AS1 was significantly increased in CCA tissues compared with the

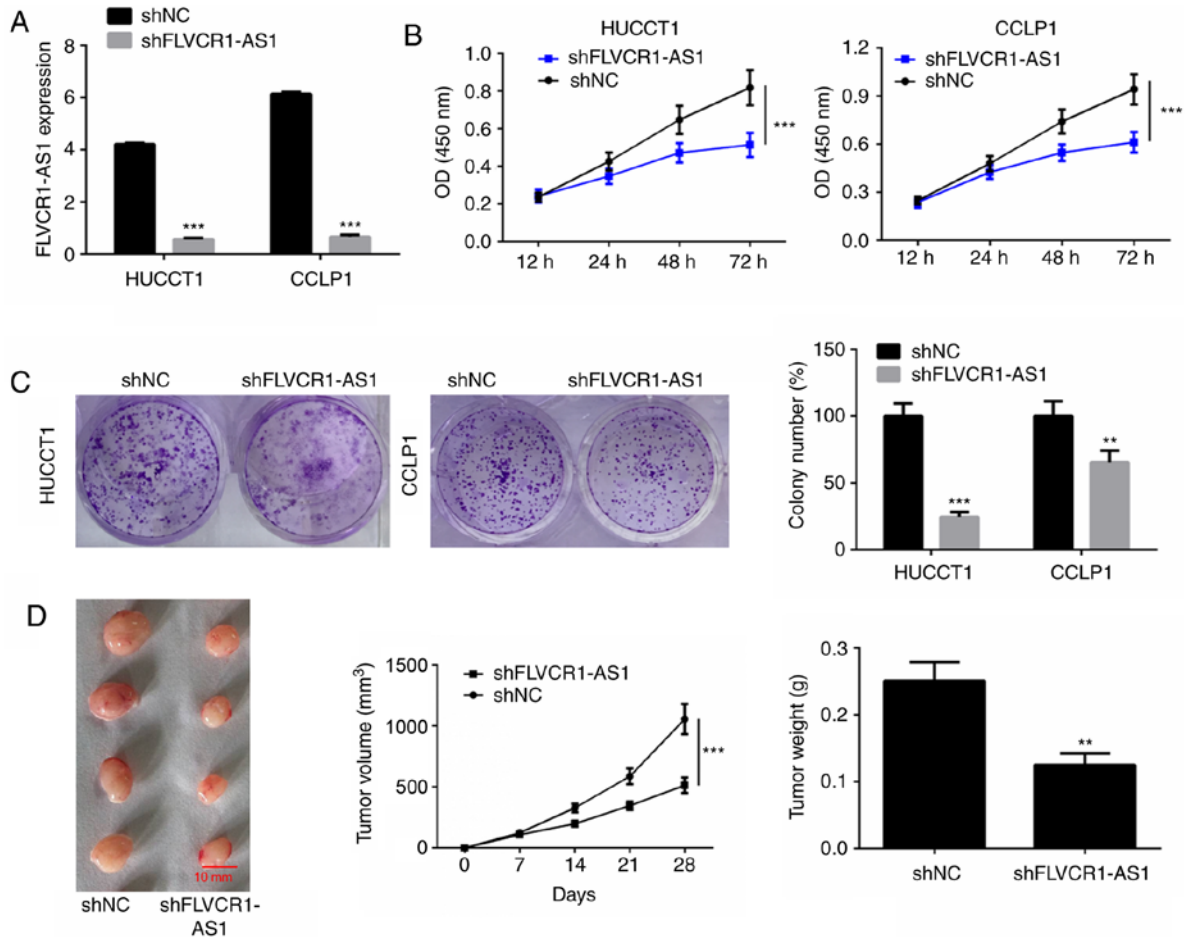

Figure 2. Downregulation of FLVCR1-AS1 inhibits cell proliferation *in vitro* and *in vivo*. (A) Relative expression level of FLVCR1-AS1 was assessed by reverse transcription-quantitative polymerase chain reaction following transfection with shFLVCR1-AS1 or shNC plasmids. (B) Cell proliferation was evaluated by MTT assay following transfection with shFLVCR1-AS1 or shNC. (C) Cell cloning capabilities of HuCCT1 and CCLP1 cells transfected with shFLVCR1-AS1 or shNC were evaluated by colony formation assay, magnification, x100. (D) Tumor growth was identified by measuring tumor volume every 7 days for 28 days after injection (n=4/group). Tumor weights isolated from nude mice in each treatment group were determined on day 28 after injection (n=4/group). Data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. shNC. FLVCR1-AS1, FLVCR1 antisense RNA 1; sh, short hairpin; NC, negative control; OD, optical density.

adjacent normal tissues. The expression of FLVCR1-AS1 was further upregulated in CCA cell lines. These results indicate that FLVCR1-AS1 may serve a role in the development and progression of CCA. CCA cell lines (HuCCT1 and CCLP1) stably transfected with shFLVCR1-AS1 or shNC were established, and cell proliferation and colony formation assays were performed. Silencing of FLVCR1-AS1 significantly inhibited cell proliferation and colony formation. Furthermore, FLVCR1-AS1-knockdown significantly inhibited tumor growth in a xenograft model, which supports the *in vitro* data. Therefore, silencing FLVCR1-AS1 was demonstrated to inhibit tumor growth *in vitro* and *in vivo*. Immoderate tumor cell migration and invasion promote cancer metastasis (25). The effects of FLVCR1-AS1-knockdown on cell migration and invasion were evaluated by wound-healing and Transwell assays, respectively. Knockdown of FLVCR1-AS1 significantly weakened the migration and invasion abilities of CCA cells. Additionally, increased expression levels of Twist, MMP-2 and MMP-9 serve important roles in the development of malignant tumors (26,27), and the expression levels of these proteins were identified to be significantly reduced following silencing of FLVCR1-AS1.

A previous study indicated that FLVCR1-AS1 promotes cancer cell proliferation, migration and invasion by sponging miR-513c in hepatocellular carcinoma (24). To elucidate the underlying molecular mechanisms by which FLVCR1-AS1 serves a tumor-promoting role in CCA, bioinformatics analysis and a luciferase reporter assay were performed. The analysis and reporter assay revealed that FLVCR1-AS1 competitively bound to miR-485-5p in CCA cells. miR-485-5p has been reported to act as a negative regulator in the progression of gastric and breast cancer (21,22). Previous studies have indicated that miR-485-5p exerts its antitumor effect by targeting nucleoside diphosphate linked moiety X, peroxisome proliferator-activated receptor-g coactivator-1α, flotillin-1 and survivin (28-30). In the present study, the expression of miR-485-5p in CCA tissues was significantly decreased compared with the normal tissues. Furthermore, FLVCR1-AS1 expression was identified to be inversely correlated with miR-485-5p expression in the CCA tissues.

In summary, lncRNA FLVCR1-AS1 was revealed to be significantly upregulated in CCA tissues and cell lines, and was demonstrated to function as an oncogene in CCA by

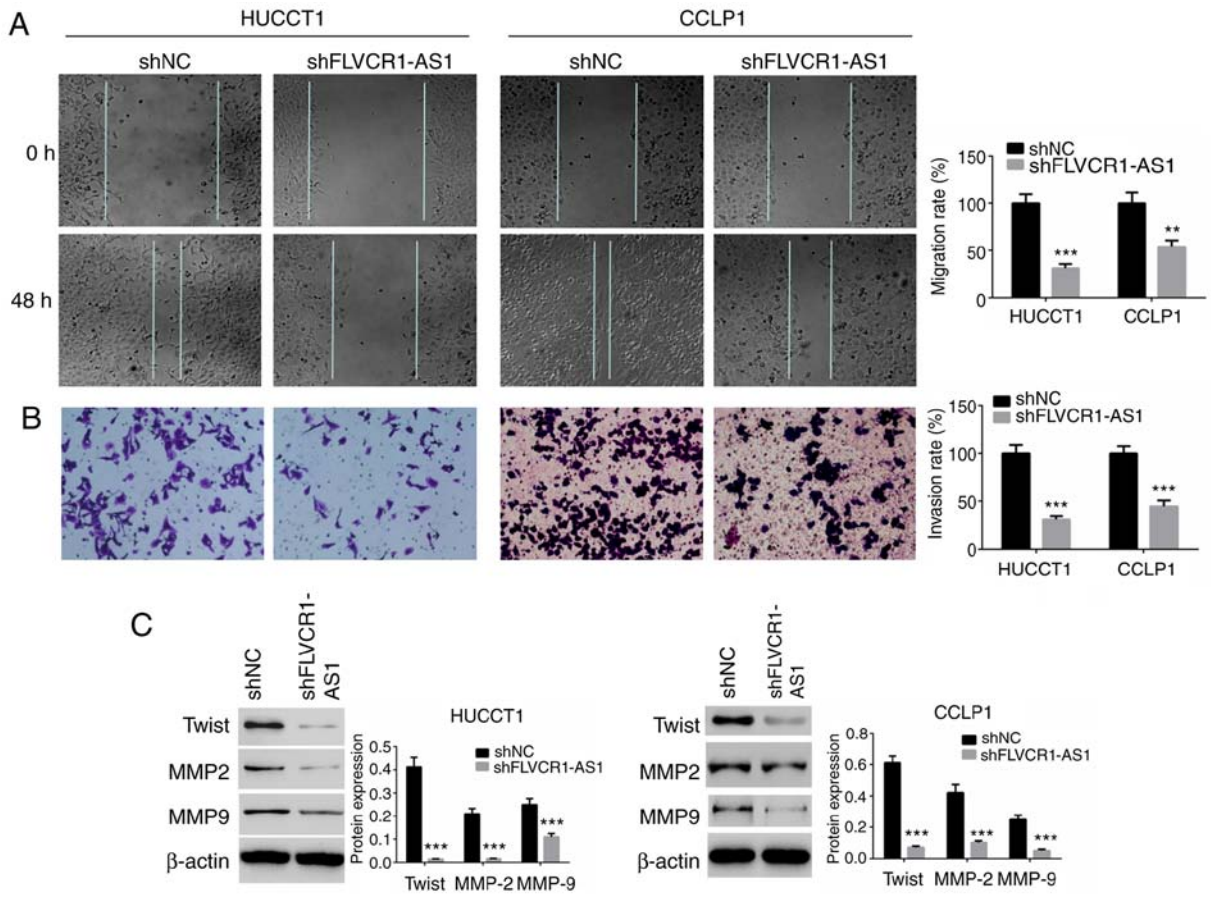

Figure 3. Silencing of FLVCR1-AS1 inhibits cell migration and invasion. (A) Cell migration was assessed by wound-healing assay and (B) invasion was evaluated by Transwell assay, magnification, x100. (C) Protein expression levels of Twist, MMP-2 and MMP-9 were determined by western blot analysis and quantified. Data are presented as the mean ± standard deviation. **P<0.01, ***P<0.001 vs. shNC. FLVCR1-AS1, FLVCR1 antisense RNA 1; sh, short hairpin; NC, negative control; MMP, matrix metalloproteinase.

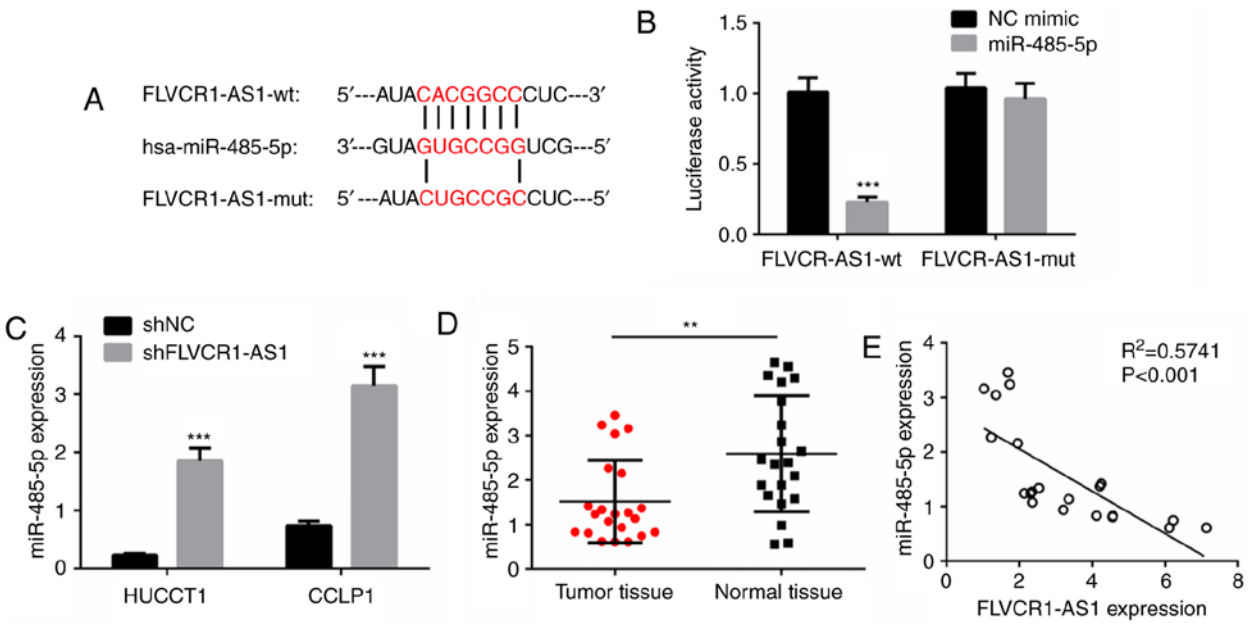

Figure 4. FLVCR1-AS1 sponges miR-485-5p in CCA cells. (A) A putative binding site of miR-485-5p in FLVCR1-AS1 was predicted using miRanda. (B) Luciferase activity was measured following co-transfection with miR-485-5p mimic and wt-FLVCR1-AS1 or mut-FLVCR1-AS1. ***P<0.001 vs. NC mimic. (C) miR-485-5p expression in HuCCT1 and CCLP1 cells transfected with shFLVCR1-AS1 or shNC was detected by RT-qPCR. ***P<0.001 vs. shNC. (D) miR-485-5p expression level in CCA and normal tissues was analyzed by RT-qPCR. **P<0.01. (E) Correlation between FLVCR1-AS1 and miR-485-5p expression in CCA tissues was determined by Pearson's correlation analysis. Data are presented as the mean ± standard deviation. FLVCR1-AS1, FLVCR1 antisense RNA 1; sh, short hairpin; NC, negative control; miR-485-5p, microRNA-485-5p; wt, wild-type; mut, mutant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CCA, cholangiocarcinoma.

sponging miR-485-5p. The present study provided novel insights that may promote understanding of the molecular mechanism of lncRNA FLVCR1-AS1 in CCA tumorigenesis and metastasis. Investigating other downstream targets of lncRNA FLVCR1-AS1 may be the focus of future studies. lncRNA FLVCR1-AS1 may serve as a potential therapeutic target and a novel diagnostic marker for CCA.

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Not applicable.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors (WB, FC, SN, HL, JY, ZS and BZ) were involved in the conception and design of this study. SN and HL performed the literature search and data extraction. SN, HL, FC, JY and ZS analyzed the data. BZ drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of human tissues was approved by the Ethics Committee of Shanghai Seventh People's Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) and written informed consent was obtained from all patients. All animal experiments were performed in accordance with institutional and international animal regulations. The animal experimental protocol was approved by the Animal Care and Use Committee of Shanghai Seventh People's Hospital affiliated to Shanghai University of Traditional Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yang J, Liu Y, Wang B, Lan H, Liu Y, Chen F, Zhang J and Luo J: Sumoylation in p27kip1 via RanBP2 promotes cancer cell growth in cholangiocarcinoma cell line QBC939. BMC Mol Biol 18: 23, 2017.
2. Sha M, Jeong S and Xia Q: Antiviral therapy improves survival in patients with HBV infection and intrahepatic cholangiocarcinoma undergoing liver resection: Novel concerns. J Hepatol 68: 1315-1316, 2018.
3. Jin Y, Gao J, Weng Q, Xiong F, Gu S, Shivaram G, Zhang F and Yang X: Cholangiocarcinoma: Molecular imaging-guided radiofrequency hyperthermia-enhanced intratumoral herpes simplex virus thymidine kinase gene therapy. Am J Cancer Res 8: 502-513, 2018.
4. Suksawat M, Techasen A, Namwat N, Boonsong T, Titapun A, Ungarreevittaya P, Yongvanit P and Loilome W: Inhibition of endothelial nitric oxide synthase in cholangiocarcinoma cell lines-a new strategy for therapy. FEBS Open Bio 8: 513-522, 2018.
5. Yoshikawa D, Ojima H, Kokubu A, Ochiya T, Kasai S, Hirohashi S and Shibata T: Vandetanib (ZD6474), an inhibitor of VEGFR and EGFR signalling, as a novel molecular-targeted therapy against cholangiocarcinoma. Br J Cancer 100: 1257-1266, 2009.
6. Chiu HS, Somvanshi S, Patel E, Chen TW, Singh VP, Zorman B, Patil SL, Pan Y, Chatterjee SS; Cancer Genome Atlas Research Network, *et al*: Pan-cancer analysis of lncRNA regulation supports their targeting of cancer genes in each tumor context. Cell Rep 23: 297-312.e12, 2018.
7. Vrba L and Futscher BW: Epigenetic silencing of lncRNA MORT in 16 TCGA cancer types. F1000Res 7: 211, 2018.
8. Zhang G, Pian C, Chen Z, Zhang J, Xu M, Zhang L and Chen Y: Identification of cancer-related miRNA-lncRNA biomarkers using a basic miRNA-lncRNA network. PLoS One 13: e196681, 2018.
9. Lou Y, Jiang H, Cui Z, Wang X, Wang L and Han Y: Gene microarray analysis of lncRNA and mRNA expression profiles in patients with high-grade ovarian serous cancer. Int J Mol Med 42: 91-104, 2018.
10. Sun T, Du SY, Armenia J, Qu F, Fan J, Wang X, Fei T, Komura K, Liu SX, Lee GM and Kantoff PW: Expression of lncRNA MIR222HG co-transcribed from the miR-221/222 gene promoter facilitates the development of castration-resistant prostate cancer. Oncogenesis 7: 30, 2018.
11. Wang H, Huo X, Yang XR, He J, Cheng L, Wang N, Deng X, Jin H, Wang N, Wang C, *et al*: STAT3-mediated upregulation of lncRNA HOXD-AS1 as a ceRNA facilitates liver cancer metastasis by regulating SOX4. Mol Cancer 16: 136, 2017.
12. Lv L, Wei M, Lin P, Chen Z, Gong P, Quan Z and Tang Z: Integrated mRNA and lncRNA expression profiling for exploring metastatic biomarkers of human intrahepatic cholangiocarcinoma. Am J Cancer Res 7: 688-699, 2017.
13. Guo L, Zhou Y, Chen Y, Sun H, Wang Y and Qu Y: LncRNA ASAP1-IT1 positively modulates the development of cholangiocarcinoma via hedgehog signaling pathway. Biomed Pharmacother 103: 167-173, 2018.
14. Xu Y, Yao Y, Jiang X, Zhong X, Wang Z, Li C, Kang P, Leng K, Ji D, Li Z, *et al*: SP1-induced upregulation of lncRNA SPRY4-IT1 exerts oncogenic properties by scaffolding EZH2/LSD1/DNMT1 and sponging miR-101-3p in cholangiocarcinoma. J Exp Clin Cancer Res 37: 81, 2018.
15. Shi X, Zhang H, Wang M, Xu X, Zhao Y, He R, Zhang M, Zhou M, Li X, Peng F, *et al*: LncRNA AFAP1-AS1 promotes growth and metastasis of cholangiocarcinoma cells. Oncotarget 8: 58394-58404, 2017.
16. Zhang S, Xiao J, Chai Y, Du YY, Liu Z, Huang K, Zhou X and Zhou W: LncRNA-CCAT1 promotes migration, invasion, and EMT in intrahepatic cholangiocarcinoma through suppressing miR-152. Dig Dis Sci 62: 3050-3058, 2017.
17. Zhang C, Li JY, Tian FZ, Zhao G, Hu H, Ma YF and Yang YL: LncRNA NEAT1 promotes growth and metastasis of cholangiocarcinoma cells. Oncol Res 26: 879-888, 2018.
18. Jiang XM, Li ZL, Li JL, Zheng WY, Li XH, Cui YF and Sun DJ: LncRNA CCAT1 as the unfavorable prognostic biomarker for cholangiocarcinoma. Eur Rev Med Pharmacol Sci 21: 1242-1247, 2017.
19. Igami T, Ebata T, Yokoyama Y, Sugawara G, Takahashi Y and Nagino M: Staging of peripheral-type intrahepatic cholangiocarcinoma: Appraisal of the new TNM classification and its modifications. World J Surg 35: 2501-2509, 2011.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Lou C, Xiao M, Cheng S, Lu X, Jia S, Ren Y and Li Z: MiR-485-3p and miR-485-5p suppress breast cancer cell metastasis by inhibiting PGC-1α expression. Cell Death Dis 7: e2159, 2016.
22. Jing LL and Mo XM: Reduced miR-485-5p expression predicts poor prognosis in patients with gastric cancer. Eur Rev Med Pharmacol Sci 20: 1516-1520, 2016.

23. Zheng B, Jeong S, Zhu Y, Chen L and Xia Q: miRNA and lncRNA as biomarkers in cholangiocarcinoma(CCA). Oncotarget 8: 100819-100830, 2017.
24. Zhang K, Zhao Z, Yu J, Chen W, Xu Q and Chen L: LncRNA FLVCR1-AS1 acts as miR-513c sponge to modulate cancer cell proliferation, migration, and invasion in hepatocellular carcinoma. J Cell Biochem 119: 6045-6056, 2018.
25. Liu B, Yan S, Jia Y, Ma J, Wu S, Xu Y, Shang M and Mao A: TLR2 promotes human intrahepatic cholangiocarcinoma cell migration and invasion by modulating NF-κB pathway-mediated inflammatory responses. FEBS J 283: 3839-3850, 2016.
26. Ince AT, Yildiz K, Gangarapu V, Kayar Y, Baysal B, Karatepe O, Kemik AS and Şentürk H: Serum and biliary MMP-9 and TIMP-1 concentrations in the diagnosis of cholangiocarcinoma. Int J Clin Exp Med 8: 2734-2740, 2015.
27. Wang Q, Tang H, Yin S and Dong C: Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9. Oncol Rep 29: 2046-2052, 2013.
28. Wang M, Cai WR, Meng R, Chi JR, Li YR, Chen AX, Yu Y and Cao XC: miR-485-5p suppresses breast cancer progression and chemosensitivity by targeting survivin. Biochem Biophys Res Commun 501: 48-54, 2018.
29. Duan J, Zhang H, Li S, Wang X, Yang H, Jiao S and Ba Y: The role of miR-485-5p/NUDT1 axis in gastric cancer. Cancer Cell Int 17: 92, 2017.
30. Kang M, Ren MP, Zhao L, Li CP and Deng MM: miR-485-5p acts as a negative regulator in gastric cancer progression by targeting flotillin-1. Am J Transl Res 7: 2212-2222, 2015.