lncRNA FLVCR1-AS1 drives colorectal cancer progression via modulation of the miR-381/RAP2A axis

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Abstract. Colorectal cancer (CRC) is one of the most prevalent types of cancer globally. Long non-coding RNAs (lncRNAs) have been suggested to serve as vital regulators in CRC. lncRNA feline leukemia virus subgroup C receptor 1 antisense RNA 1 (FLVCR1-AS1) is closely associated with the tumorigenesis of various types of cancer. The aim of the present study was to investigate the molecular mechanisms of lncRNA FLVCR1-AS1 in CRC progression. The expression levels of FLVCR1-AS1, microRNA (miR)-381 and Ras-related protein 2a (RAP2A) were measured by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A Kaplan-Meier analysis was performed to determine the overall survival rate of patients with CRC. Furthermore, cell viability, migration and invasion were assessed using Cell Counting Kit-8 (CCK-8) and Transwell assays. The interaction between genes was confirmed using dual-luciferase reporter and pull-down assays. The results demonstrated that FLVCR1-AS1 was upregulated in CRC tissues and cells, and increased FLVCR1-AS1 expression levels in patients with CRC were associated with poor prognosis. FLVCR1-AS1 knockdown significantly attenuated the viability, migration and invasion ability of CRC cells. In addition, the results confirmed that FLVCR1-AS1 directly binds with miR-381-3p, and that RAP2A is a direct target of miR-381-3p. The overexpression of FLVCR1-AS1 increased RAP2A expression levels. Functional assays revealed that miR-381 inhibitor or RAP2A overexpression attenuated the suppressive effects of FLVCR1-AS1 silencing on CRC cell viability, migration and invasion. Overall, the findings of the current study suggest that FLVCR1-AS1 promotes CRC progression via the miR-381/RAP2A pathway. These findings may provide a novel approach for CRC treatment.

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Introduction

Colorectal cancer (CRC) is one of the most common types of cancer and has high incidence and mortality rates (1,2). Despite advancements in the clinical treatments for patients with CRC, the overall survival (OS) rate has not improved due to recurrence and metastasis (3). Therefore, it is urgently necessary to identify new biomarkers for the malignant progression of CRCs.

Long non-coding RNAs (lncRNAs) are RNAs >200 nucleotides in length that do not have protein-coding capacity (4). Numerous studies have reported that lncRNAs are involved in the regulation of biological behaviors of tumors, including proliferation, apoptosis and metastasis (5,6). For example, lncRNA Down syndrome cell adhesion molecule antisense RNA 1 (DSCAM-AS1) facilitates osteosarcoma cell metastasis by sponging microRNA (miR)-101-3p (7). In addition, it has been reported that the lncRNA wee1-like protein kinase 2 antisense RNA 1 (WEE2-AS1) suppresses breast cancer cell apoptosis by modulating transducer of ERBB2, 1 (TOB1) and sponging miR-32-5p (8), whereas LINC01535 promotes the development of esophageal squamous cell cancer by regulating the JAK/STAT3 pathway (9). The present study aimed to investigate the role of lncRNAs in CRC progression. Previous studies have revealed that feline leukemia virus subgroup C receptor 1 antisense RNA 1 (FLVCR1-AS1) serves an oncogenic role in the development of several types of tumors. For instance, it has been demonstrated that lncRNA FLVCR1-AS1 promotes glioma by modulating the miR-4731-5p/E2F transcription factor 2 (E2F2) axis (10), and contributes to the malignant progression of ovarian cancer by sponging miR-513 (11). Furthermore, it has been shown that silencing lncRNA FLVCR1-AS1 represses the proliferation of lung cancer cells by regulating the Wnt/β-catenin pathway (12). Nevertheless, the mechanism of FLVCR1-AS1 in CRC remains unclear.

Numerous studies have indicated that lncRNAs exert their functions via the targeting of miRNAs (13-15). miRNAs have been reported to have tumor suppressive functions in multiple human cancers. For instance, studies have shown that miRNA-339-5p inhibits gastric cancer development via the upregulation of alkB homolog 1 (ALKBH1) (16), miR-95-3p regulates CRC cell proliferation via the downregulation of hepatoma-derived growth factor (HDGF) (17), and miR-30a-3p

represses renal cancer cell metastasis via the regulation of autophagy-related protein 12 (ATG12) expression (18). Furthermore, several studies have shown that miR-381 is associated with the development of different types of cancer, including cervical (19), gastric (20) and prostate cancer (21). A previous research has reported that miR-381 acted as a tumor suppressor in CRC by targeting Twist1 (22). However, the regulatory mechanism of miR-381 in CRC needs to be further elucidated.

The current study aimed to evaluate the FLVCR1-AS1 expression in CRC, and to investigate the biological role of FLVCR1-AS1 in modulating the malignant phenotypes of CRC cells *in vitro*.

Materials and methods

The Cancer Genome Atlas (TCGA) analysis. The expression of FLVCR1-AS1 and CRC clinical data were downloaded from the TCGA database (https://tcga-data.nci.nih.gov/tcga/). Kaplan-Meier analysis and the log-rank test were used to analyze survival curves. Cut-off values were determined using mean expression level of FLVCR1-AS1.

Tissue specimens. A total of 26 pairs of CRC tissues and adjacent non-tumor tissues were obtained from patients with CRC (14 males and 12 females) with a mean age of 52 years (range, 34-82 years) between April 2017 and September 2018 at Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine. Written informed consent was obtained from all subjects and the study was approved by the Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine. The specimens were immediately cryopreserved in liquid nitrogen after surgery.

Cell lines and cell culture. Four human CRC cell lines, namely Caco-2, SW480, LoVo and SW1116, the NCM460 normal colonic epithelial cell line and 293T cells were purchased from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humified atmosphere containing 5% CO_2.

Cell transfection. Short hairpin RNA (shRNA) targeting FLVCR1-AS1 (shFLVCR1-AS1) and its corresponding control shRNA (shNC), miR-381 mimics, 5'-UACAGUACUGUGAUAACUGAA-3'; NC mimics, 5'UUUGUACUACACAAAAGUACUG-3'; miR-381 inhibitor, 5'-UUCAGUUAUCACAGUACUGUA-3'; NC inhibitor, 5'-UCACAACCUCCUAGAAAGAGUAGA-3'. The full-length of FLVCR1-AS1 and RAP2A were subcloned into pcDNA3.1 to overexpress FLVCR1-AS and RAP2A levels, with empty pcDNA3.1 serving as control. The overexpression plasmid and pcDNA3.1 were bought from Shanghai GenePharma Co., Ltd. shRNA (100 nm), miRNA (50 nM) or plasmid (1 μg/ml) was transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequent experiments were performed at 48 h post-transfection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) at 37˚C for 15 min. RT-qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SYBR-Green PCR Master Mix kit (Takara Bio, Inc.). The PCR amplification reaction was carried out using cDNA as template with the following conditions: 95˚C for 10 min, and 40 cycles of 95˚C for 15 sec, 62˚C for 30 sec, and 72˚C for 30 sec. Primers were as follows: FLVCR1-AS1 forward, 5'-GAAAGCTGCAACATGCTCCC-3' and reverse, 5'-TCCATGTCGTCCCAGTTGGT-3'; miR-381 forward, 5'-GCAGGGCTTCTGAGCTCCTTAA-3' and reverse, 5'-CAAATTCGTGAAGCGTTCCATAT-3'; RAP2A forward, 5'-ACACGTCTGGAGGAGACAGC-3' and reverse, 5'-GAGAGGTTGTGCCGGATAGA-3'; GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The relative expression of genes was calculated using the $2^{-\Delta\Delta Cq}$ method (23), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as internal references.

Cell proliferation. Cell viability was evaluated using a Cell Counting Kit 8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. Briefly, $2x10^4$ cells/well were seeded into 96-well plates and 48 h following transfection, each well was supplemented with 10 μl CCK-8 reagent. Subsequently, the optical density value at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

TUNEL assay. An *in situ* Cell Death Detection kit (cat. no. 11684817910; Roche Diagnostics GmbH) was used to analyze cell apoptosis. Caco-2 and SW480 cells were washed with PBS, and fixed in 4% paraformaldehyde solution for 1 h at 4˚C. The cells were permeabilized in a solution containing 0.1% Triton X-100 for 2 min and cultured with TUNEL reaction mixture (Roche Diagnostics GmbH) for 1 h at 37˚C in the dark. The TUNEL-stained coverslips were then washed with PBS and then counterstained with DAPI (1:20 dilution; Beyotime Institute of Biotechnology) for 15 min at room temperature. A fluorescence microscope (magnification, x100; Olympus Corporation) was utilized to observe TUNEL-positive cells in at least 5 fields of view.

Bioinformatics analysis and dual-luciferase reporter assay. The starBase database (http://starbase.sysu.edu.cn/) were used for predicting the binding sites between miR-381 and FLVCR1-AS1 or RAP2A. The wild-type (wt) or mutant (mut) FLVCR1-AS1/RAP2A was inserted into a pGL3.0 vector (Shanghai GenePharma Co., Ltd.). Subsequently, 293T cells were transfected with one of the above vectors and either miR-381 mimics or NC mimics using Lipofectamine 2000. Following incubation for 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase gene activity.

Pull-down assay. Biotin-labeled wt miR-381 (bio-miR-381-wt), mut miR-381 (bio-miR-381-mut) and miR-NC were obtained

from GenePharma Co., Ltd. The biotinylated RNAs were transfected into Caco-2 and SW480 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), and the cell lysates were incubated with streptavidin magnetic beads (Invitrogen; Thermo Fisher Scientific, Inc.) overnight. Following RNA extraction, the abundance of FLVCR1-AS1 was detected by RT-qPCR.

Transwell assay. Cell migration and invasion abilities were assessed using Transwell chambers (8.0 μm pore size; BD Biosciences). For cell migration, $1x10^5$ transfected cells and 200 μl serum-free DMEM were added to the upper chamber with an uncoated membrane. Then, complete DMEM was added to the lower chamber. Following seeding for 48 h, cells in the upper chamber were removed, and those in the lower chamber were stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA). For cell invasion, the membranes were precoated with Matrigel at 37˚C for 2 h, and the other steps were the same as those carried out in the migration assay. Cell numbers were counted under a light microscope. (magnification, x200; Zeiss GmbH).

Western blot assay. Total protein extracts were isolated from transfected cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 10 μg protein/lane was separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc.). The proteins were then electrotransferred onto a PVDF membrane. Following blocking with non-fat milk for 2 h at room temperature, membranes were first incubated with primary antibodies against RAP2A (1:1,000; cat. no. ab101369; Abcam) and GAPDH (1:1,000; cat. no. ab9485; Abcam) at 4˚C overnight and then with corresponding secondary antibodies (1:1,000; goat anti-rabbit IgG, cat. no. ab205718; Abcam) for 1 h at room temperature. Finally, blots were visualized with an enhanced chemiluminescent detection system (EMD Millipore). Protein expression was evaluated using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc.).

Statistical analysis. SPSS 19.0 (IBM Corp.) software was used to perform the statistical analyses, and results are expressed as the mean ± standard deviation. Each experiment was performed at least three times. Clinicopathological characteristics were evaluated using the χ^2 test. Paired or unpaired Student's t-tests, or one-way ANOVA followed by Tukey's post hoc test were used to compare the groups. The survival curve was generated using the Kaplan-Meier method and log-rank test with the 'survival' R package (version 3.4.1; http://bioconductor.org/packages/survivalr/). The patients were divided into a high and a low expression group according to the mean gene expression. The correlation between gene expression levels was analyzed using Pearson's correlation coefficient. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FLVCR1-AS1 is upregulated in CRC. Firstly, the expression of FLVCR1-AS1 in CRC tissues was examined, and the results

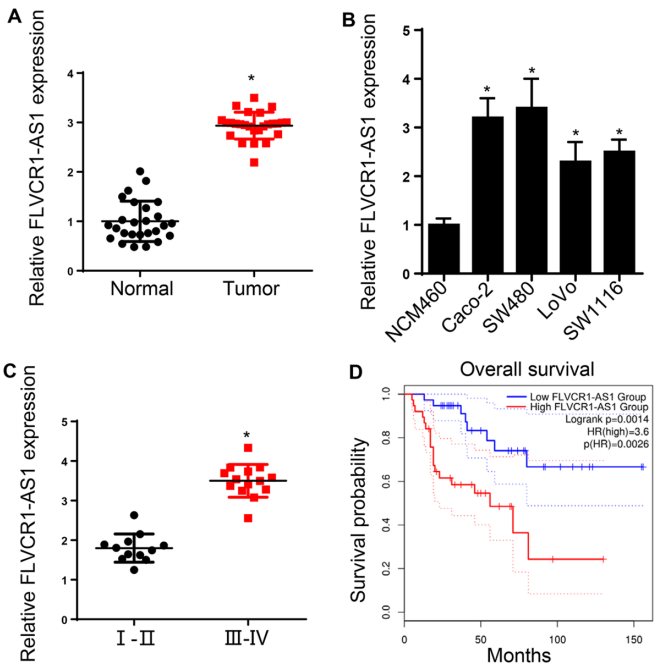

Figure 1. FLVCR1-AS1 expression is upregulated in CRC. (A) RT-qPCR results show that FLVCR1-AS1 expression is higher in CRC tissues compared with normal tissues (n=26). (B) RT-qPCR was used to measure the expression of FLVCR1-AS1 in CRC cell lines (Caco-2, SW480, LoVo and SW1116) and the NCM460 normal colonic epithelial cell line. (C) RT-qPCR results showing the level of FLVCR1-AS1 in CRC tissues of different TNM stages. *P<0.05. (D) Kaplan-Meier analysis reveals the association of high FLVCR1-AS1 expression with poor overall survival in patients with CRC. FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

indicated that the FLVCR1-AS1 expression levels were significantly elevated in CRC tissues compared with the adjacent normal tissue (Fig. 1A). Consistent with this, FLVCR1-AS1 was found to be upregulated in CRC cells (Caco-2, SW480, LoVo and SW111) compared with normal colonic epithelial cells (Fig. 1B). Analysis of the expression of FLVCR1-AS1 according to different clinicopathological characteristics of the patients with CRC indicated that high levels of FLVCR1-AS1 were positively associated with TNM stage, distant metastasis and lymph node metastasis (Table I). Notably, the expression of FLVCR1-AS1 was significantly higher in CRC tissues of advanced TNM stages compared with earlier ones (Fig. 1C). Furthermore, Kaplan-Meier analysis showed that CRC patients with high levels of FLVCR1-AS1 exhibited a shorter survival time than those with low FLVCR1-AS1 levels (Fig. 1D). These findings suggest that FLVCR1-AS1 is involved in CRC tumorigenesis.

FLVCR1-AS1 modulates CRC cell proliferation, metastasis and apoptosis. The biological role of FLVCR1-AS1 in CRC was further explored. The expression of FLVCR1-AS1 was significantly decreased in Caco-2 and SW480 cells following transfection with shFLVCR1-AS1 compared with shNC (Fig. 2A). CCK-8 and Transwell assays demonstrated that the knockdown of FLVCR1-AS1 attenuated CRC cell viability, migration and invasion (Fig. 2B-D). Furthermore, TUNEL assay revealed that FLVCR1-AS1 silencing induced

Table I. Association between clinicopathological factors and FLVCR1-AS1 expression in patients with colorectal cancer.

		lncRNA FLVCR1-AS1 expression, n		
Parameters	Total	Low	High	P-value
Sex				0.603
Female	12	6	6	
Male	14	7	7	
Age (years)				0.552
≤60	15	8	7	
>60	11	5	6	
TNM stage				0.024
I-II	12	7	5	
III-IV	14	6	8	
Tumor size (cm)				0.817
≤2	11	6	5	
>2	15	7	8	
Lymph node metastasis				0.016
Positive	13	4	9	
Negative	13	9	4	
Distant metastasis				0.033
Positive	8	3	5	
Negative	18	10	8	

FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1.

Caco-2 and SW480 cell apoptosis (Fig. 2E). Subsequently, RT-qPCR analysis showed that FLVCR1-AS1 was efficiently overexpressed following transfection with an FLVCR1-AS1 overexpression plasmid (Fig. 2F). CCK-8 and Transwell assays indicated that transfection with pcDNA3.1/FLVCR1-AS1 increased the viability, migration and invasion of Caco-2 and SW480 cells (Fig. 2G-I). In addition, TUNEL assay revealed that FLVCR1-AS1 overexpression markedly attenuated CRC cell apoptosis (Fig. 2J). The aforementioned data indicate that FLVCR1-AS1 promotes CRC progression.

FLVCR1-AS1 acts as a competing endogenous RNA (ceRNA) for miR-381 in CRC. To explore the molecular mechanism of FLVCR1-AS1 in CRC, its downstream targets were identified. Using the starBase online tool, the binding sequence between miR-381 and FLVCR1-AS1 was predicted (Fig. 3A). RT-qPCR analysis showed that miR-381 expression was successfully elevated in Caco-2 and SW480 cells following transfection with miR-381 mimics compared with NC mimics (Fig. 3B). A luciferase reporter assay indicated that the luciferase activity of FLVCR1-AS1-wt was significantly inhibited by miR-381 mimics. However, miR-381 mimics had no effect on the luciferase activity of FLVCR1-AS1-mut in 293T cells (Fig. 3C). Moreover, the RNA pull-down assay demonstrated that the FLVCR1-AS1 expression levels were elevated in Caco-2 and SW480 cells following transfection with bio-miR-381-wt (Fig. 3D). Furthermore, RT-qPCR analysis revealed that the expression of miR-381 was downregulated in CRC tissues compared with adjacent normal tissues (Fig. 3E), and the knockdown of FLVCR1-AS1 increased miR-381 expression in Caco-2 and SW480 cells (Fig. 3F). Following confirmation by RT-qPCR analysis that miR-381 was successfully knocked down in Caco-2 and SW480 cells following transfection with miR-381 inhibitor (Fig. 3G), whether FLVCR1-AS1 promotes CRC progression by modulating miR-381 was further investigated. CRC cells were transfected with shNC, shFLVCR1-AS1 and shFLVCR1-AS1 + miR-318 inhibitor. CCK-8 and Transwell assays of the transfected cells demonstrated that the miR-381 inhibitor attenuated the inhibitory effect of FLVCR1-AS1 knockdown on CRC cell viability, migration and invasion (Fig. 3H-J). The aforementioned findings suggest that FLVCR1-AS1 may act as a molecular sponge for miR-381 in CRC.

RAP2A is directly targeted by miR-381. Bioinformatics analysis was conducted using starBase software to predict the downstream targets of miR-381. The analysis revealed that RAP2A includes a potential binding site for miR-381 (Fig. 4A). A luciferase reporter assay demonstrated that miR-381 mimics inhibit the luciferase activity of RAP2A-wt but have no effect on the luciferase activity of RAP2A-mut (Fig. 4B). In addition, RT-qPCR results showed that RAP2A was highly expressed in CRC tissues compared with adjacent normal tissue (Fig. 4C). In addition, RT-qPCR and western blot assays indicated that overexpression of miR-381 significantly reduced RAP2A expression in Caco-2 and SW480 cells (Fig. 4D). Subsequently, the association between RAP2A and FLVCR1-AS1 was evaluated. The results indicated that FLVCR1-AS1 knockdown downregulated RAP2A at the mRNA and protein levels (Fig. 4E). In addition, transfection with pcDNA3.1/FLVCR1-AS1 attenuated the miR-381-mediated inhibition of RAP2A expression (Fig. 4F). Additionally, Pearson's correlation analysis showed that the expression of miR-381 was negatively correlated with that of FLVCR1-AS1 and RAP2A in CRC tissues (Fig. 4G). The aforementioned data suggest that FLVCR1-AS1 increases RAP2A expression via the repression of miR-381 in CRC.

FLVCR1-AS1 promotes CRC progression by upregulating RAP2A. To investigate whether the molecular functions of FLVCR1-AS1 are mediated by the upregulation of RAP2A in CRC, the Caco-2 and SW480 cells were transfected with shNC, shFLVCR1-AS1 or shFLVCR1-AS1 + RAP2A, and RT-qPCR and western blot assays were then carried out. First, it was confirmed that RAP2A expression was successfully increased by transfection with RAP2A overexpression vector (Fig. 5A). The RT-qPCR and western blotting results demonstrated that RAP2A overexpression attenuated the FLVCR1-AS1 knockdown-mediated reduction of RAP2A expression (Fig. 5B). Similarly, CCK-8 and Transwell assays confirmed that RAP2A overexpression attenuated the effect of FLVCR1-AS1 downregulation on Caco-2 and SW480 cell proliferation, migration, and invasion (Fig. 5C-E). These findings indicate that FLVCR1-AS1 promotes the development of CRC by modulating the expression of RAP2A.

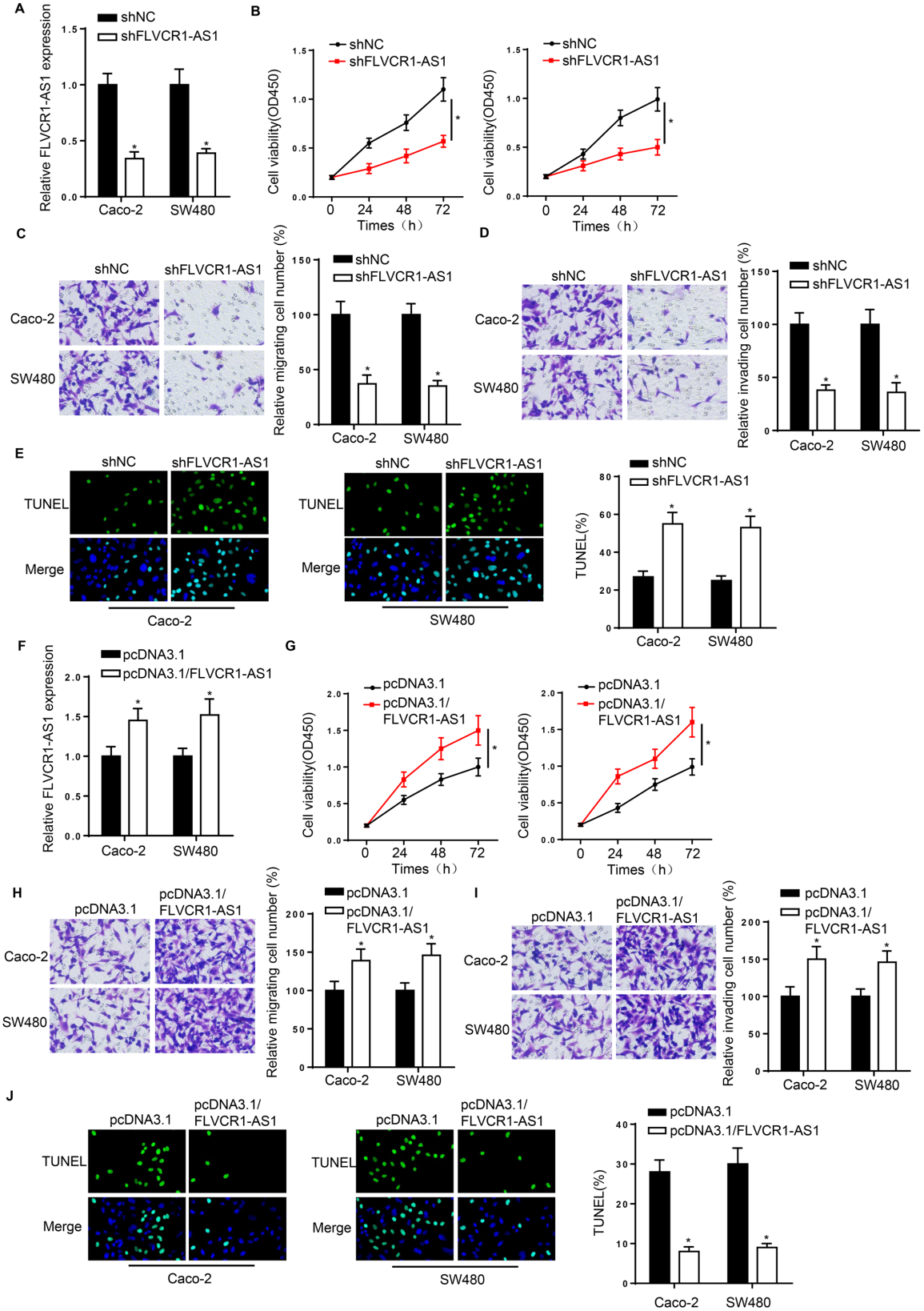

Figure 2. FLVCR1-AS1 modulates the proliferation, metastasis and apoptosis of colorectal cancer cells. (A) RT-qPCR was used to confirm the reduction of FLVCR1-AS1 expression in Caco-2 and SW480 cells transfected with shFLVCR1-AS1 compared with shNC. (B) CCK-8 and Transwell (magnification, x200) (C) migration and (D) invasion assays were performed to evaluate the proliferation, migration and invasion abilities of Caco-2 and SW480 cells transfected with shFLVCR1-AS1 and shNC. (E) TUNEL assay (magnification, x100) was used to estimate the apoptosis of Caco-2 and SW480 cells after FLVCR1-AS1 knockdown. (F) RT-qPCR was used to determine the expression of FLVCR1-AS1 in Caco-2 and SW480 cells transfected with pcDNA3.1/FLVCR1-AS1 and pcDNA3.1. (G) CCK-8 and Transwell (magnification, x200) (H) migration and (I) invasion assays were performed to evaluate the proliferation, migration and invasion abilities of Caco-2 and SW480 cells transfected with pcDNA3.1/FLVCR1-AS1 and pcDNA3.1. (J) TUNEL assay (magnification, x100) was used to estimate the apoptosis of Caco-2 and SW480 cells transfected with pcDNA3.1/FLVCR1-AS1 and pcDNA3.1. *P<0.05. FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; sh, short hairpin; NC, negative control; CCK-8, Cell Counting Kit 8; OD450, optical density at 450 nm.

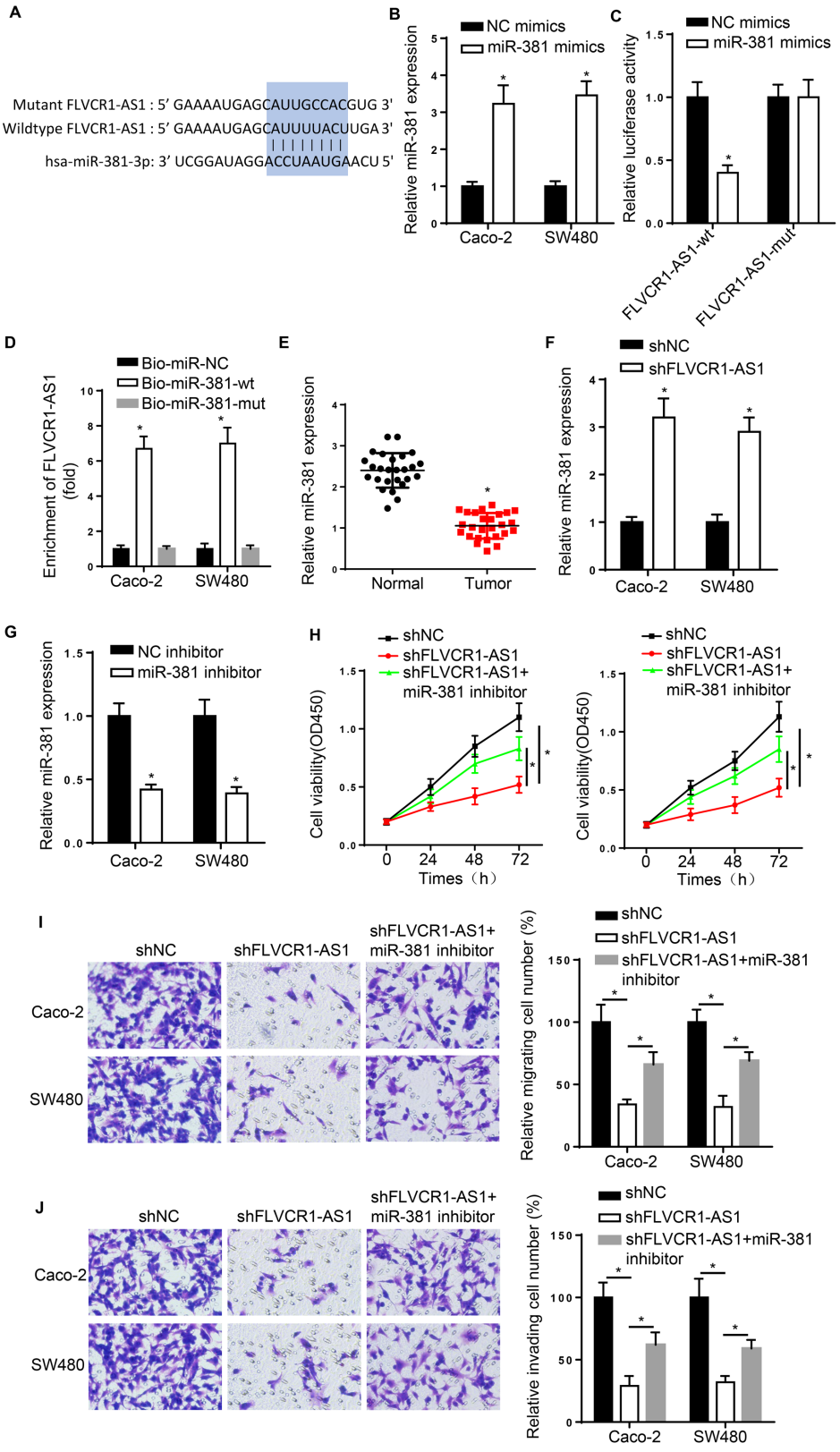

Figure 3. FLVCR1-AS1 functions as a competing endogenous RNA for miR-381 in CRC. (A) The binding sequence between FLVCR1-AS1 and miR-381 was predicted using starBase. (B) RT-qPCR results show that the level of miR-381 was successfully increased in Caco-2 and SW480 cells transfected with miR-381 mimics. (C) Luciferase reporter assay results showing the luciferase activity of FLVCR1-AS1-wt and FLVCR1-AS1-mut in 293T cells transfected with NC mimics or miR-381 mimics. *P<0.05. (D) RNA pull-down assay was used to verify the interaction between FLVCR1-AS1 and miR-381 in Caco-2 and SW480 cells transfected with bio-miR-381-wt or bio-miR-381-mut. *P<0.05 vs. Bio-miR-NC. (E) RT-qPCR assay results show that the level of miR-381 is lower in CRC tissues compared with normal tissues. (F) RT-qPCR assay results show that the expression of miR-381 is increased in cells transfected with shFLVCR1-AS1. (G) RT-qPCR results show that the level of miR-381 in Caco-2 and SW480 cells was successfully reduced by transfection with miR-381 inhibitor. (H) Cell Counting Kit-8, (I) Transwell migration assay (magnification, x200) and (J) invasion assay results demonstrating the proliferation, migration and invasion of Caco-2 and SW480 cells transfected with shNC, shFLVCR1-AS1 and shFLVCR1-AS1+ miR-381 inhibitor. *P<0.05. FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1; miR, microRNA; CRC, colorectal cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; bio, biotin; wt, wild-type; mut, mutant; sh, short hairpin; NC, negative control; OD450, optical density at 450 nm.

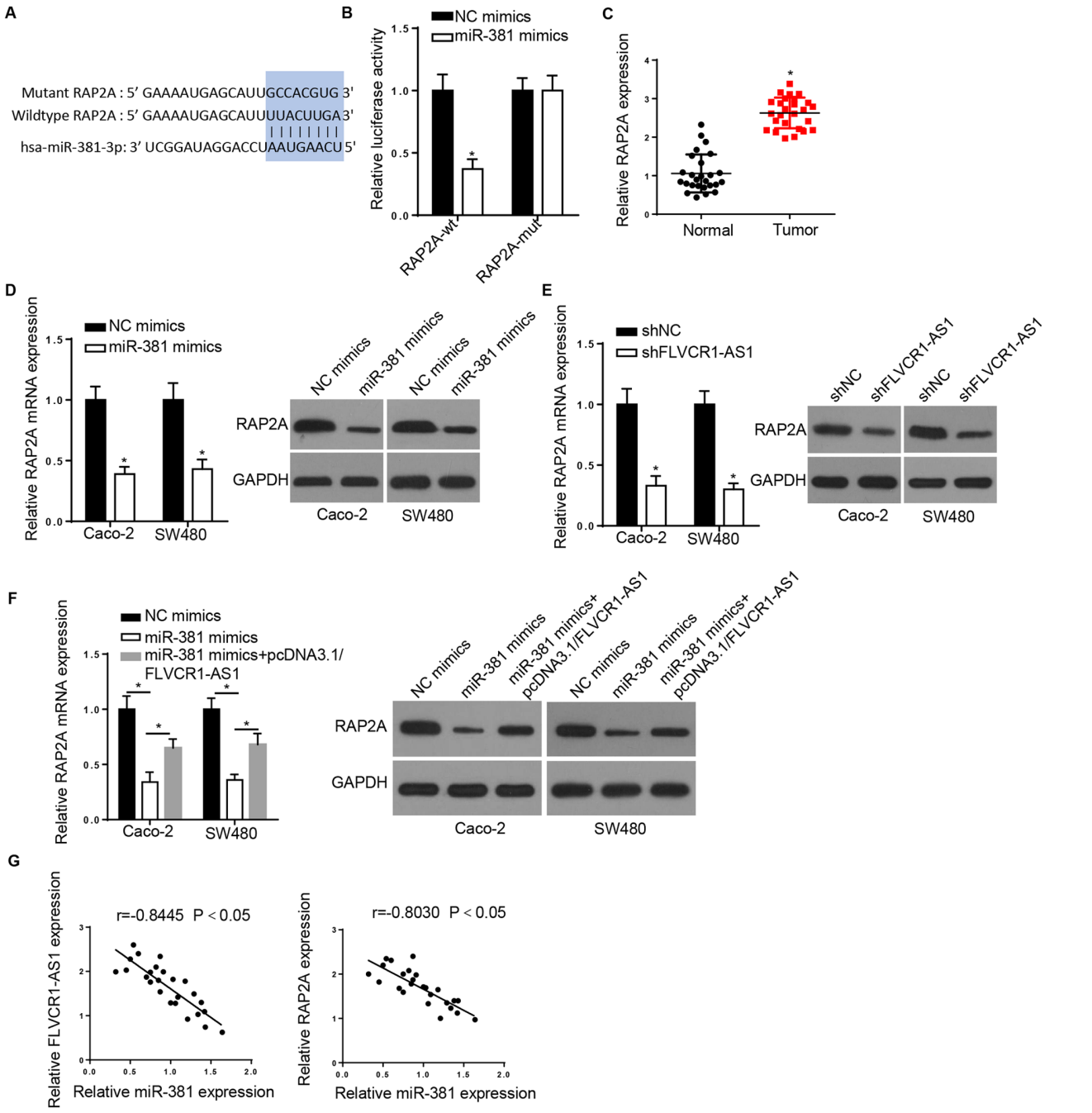

Figure 4. RAP2A is a target of miR-381. (A) Binding sequence between RAP2A and miR-381 was predicted using starBase. (B) Luciferase reporter assay showing the luciferase activity of RAP2A-wt and RAP2A-mut in 293T cells transfected with NC mimics or miR-381 mimics. (C) RT-qPCR assay results show that the level of RAP2A is higher in CRC tissues than in normal tissues. RT-qPCR and western blot assays were employed to assess RAP2A expression in Caco-2 and SW480 cells with (D) overexpression of miR-381 and (E) knockdown of FLVCR1-AS1. (F) RT-qPCR and western blot assays were performed to evaluate RAP2A expression in Caco-2 and SW480 cells transfected with miR-NC, miR-381 and miR-381 + pcDNA3.1/FLVCR1-AS1. *P<0.05. (G) Pearson's correlation analysis was used to assess the correlation of miR-381 with FLVCR1-AS1 or RAP2A in CRC tissues. RAP2A, Ras-related protein 2a; miR, microRNA; wt, wild-type; mut, mutant; sh short hairpin; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1; CRC, colorectal cancer.

Discussion

The present study demonstrated that lncRNA FLVCR1-AS1 was elevated in CRC tissues compared with adjacent normal tissues, and the upregulated expression of FLVCR1-AS1 was associated with poor prognosis in patients with CRC, thus implying its essential role in CRC progression. Previous studies have shown that the abnormal expression of FLVCR1-AS1 is associated with the tumorigenesis of multiple cancers (24,25). For example, FLVCR1-AS1 was reported to be upregulated in lung cancer and hepatocellular carcinoma, in which it promoted cell metastasis (26) and tumor growth (27), respectively. Therefore, we hypothesized that FLVCR1-AS1 could be involved in the development of CRC. The findings of the current study reveal that the downregulation of FLVCR1-AS1 attenuated CRC cell viability, migration and invasion, thus

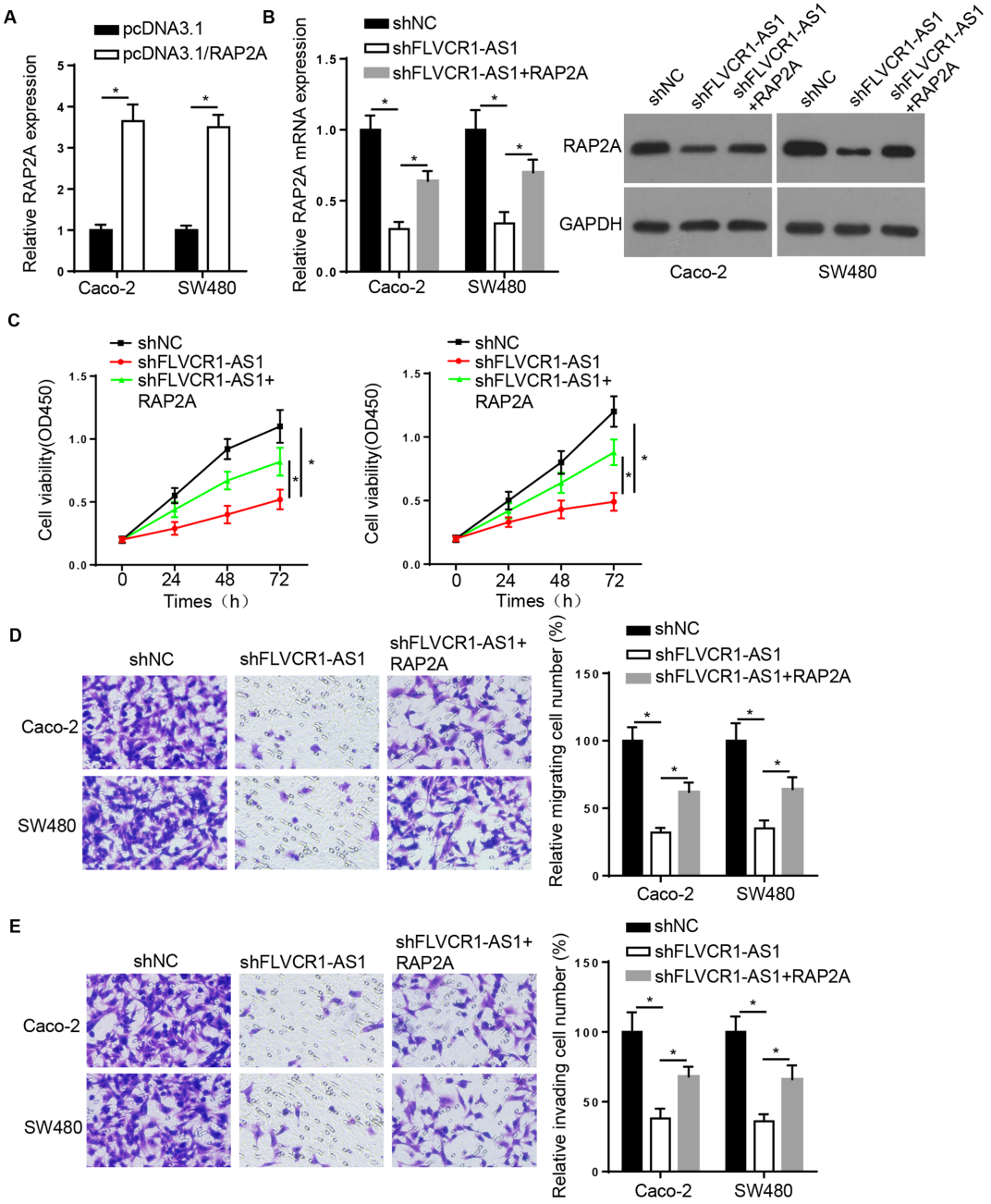

Figure 5. FLVCR1-AS1 promotes colorectal cancer progression by upregulating RAP2A. (A) RT-qPCR analysis shows that the expression of RAP2A was increased by transfection with RAP2A overexpression vector in Caco-2 and SW480 cells. (B) RT-qPCR and western blot assays were used to analyze RAP2A expression in Caco-2 and SW480 cells transfected with shNC, shFLVCR1-AS1 and shFLVCR1-AS1 + RAP2A. (C) Cell Counting Kit-8 and Transwell (magnification, x200) (D) migration and (E) invasion assays demonstrating the proliferation, migration and invasion of Caco-2 and SW480 cells transfected with shNC, shFLVCR1-AS1 and shFLVCR1-AS1 + RAP2A. *P<0.05. FLVCR1-AS1, feline leukemia virus subgroup C receptor 1 antisense RNA 1; RAP2A, Ras-related protein 2a; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; sh short hairpin; NC, negative control.

suggesting that FLVCR1-AS1 serves an oncogenic role in CRC.

There is considerable evidence that lncRNAs act as molecular sponges to modulate miRNAs (28). For example, it has been reported that lncRNA associated with poor prognosis of hepatocellular carcinoma (AWPPH) sponges miRNA-204 to promote the development of non-small cell lung cancer (29). In addition, lncRNA olfactory receptor 3A4 (OR3A4) has been shown to accelerate osteosarcoma cell proliferation via the targeting of miR-1207-5p (30). Furthermore, the miRNA-20a-5p/tripartite motif containing 32 (TRIM32) axis has been shown to be associated with the apoptosis of breast cancer cells following hepatocyte nuclear factor 1-α antisense RNA 1 (HNF1A-AS1) silencing (31). It has also been revealed that FLVCR1-AS1 acts as a ceRNA that is involved in the pathogenesis and development of several types of cancer. Bao *et al* (24) demonstrated that lncRNA FLVCR1-AS1 acts as a sponge for miR-573 and thereby regulates cholangiocarcinoma cell proliferation. Liu *et al* (25) suggested that lncRNA FLVCR1-AS1 acts as an oncogene in gastric cancer via regulation of the miR-155/c-Myc pathway. The present study identified miR-381 as a novel target of FLVCR1-AS1, and

transfection with miR-381 inhibitor attenuated the suppressive effect of FLVCR1-AS1 silencing on CRC cell viability and metastasis. These findings indicate that miR-381 exerts an antitumor effect on CRC.

Furthermore, RAP2A was identified as a direct target of miR-381 in the present study. Several studies have reported that RAP2A may be used as a therapeutic biomarker and acts as an oncogene in various cancers. For example, Zhang *et al* (32) revealed that the overexpression of RAP2A promoted gastric cancer progression, and another study demonstrated that it accelerated the metastasis and growth of hepatocellular carcinoma cells (33). In addition, RAP2A has been identified to be contribute to the progression of tumors via the regulation of the phosphorylation of AKT. For instance, one study showed that RAP2A promoted the migration and invasion of renal cancer cells by increasing phosphorylated (p)-AKT levels (34), while another demonstrated that the overexpression of RAP2A increased cancer cell migration, invasion and metastasis by the upregulation of p-AKT (35). Consistent with previous studies, the current study revealed that the expression of RAP2A was markedly increased in CRC tissues, and its expression was regulated by the FLVCR1-AS1/miR-381 axis. Moreover, RAP2A overexpression reversed the inhibitory effect of FLVCR1-AS1 silencing on CRC cell viability, apoptosis, migration and invasion, thus supporting the oncogenic role of RAP2A in CRC.

In conclusion, the present study suggests that FLVCR1-AS1 acts as a ceRNA to increase RAP2A expression by sponging miR-381, thereby facilitating CRC tumorigenesis. However, certain limitations remain to be addressed in future studies. In particular, the biological roles of FLVCR1-AS1 in CRC *in vivo*, and the downstream effectors of RAP2A require further investigation.

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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 on reasonable request.

Authors' contributions

YH, XW and LH designed the present study. EM and BS performed the experiments. LH and BS analyzed the data and prepared the figures. YH and XW drafted the initial manuscript. EM and LH reviewed and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2019. CA Cancer J Clin 69: 7-34, 2019.
2. Ahnen DJ, Wade SW, Jones WF, Sifri R, Mendoza Silveiras J, Greenamyer J, Guiffre S, Axilbund J, Spiegel A and You YN: The increasing incidence of young-onset colorectal cancer: A call to action. Mayo Clin Proc 89: 216-224, 2014.
3. Ladabaum U, Dominitz JA, Kahi C and Schoen RE: Strategies for colorectal cancer screening. Gastroenterology 158: 418-432, 2020.
4. Uszczynska-Ratajczak B, Lagarde J, Frankish A, Guigó R and Johnson R: Towards a complete map of the human long non-coding RNA transcriptome. Nat Rev Genet 19: 535-548, 2018.
5. Davalos V and Esteller M: Disruption of long noncoding RNAs targets cancer hallmark pathways in lung tumorigenesis. Cancer Res 79: 3028-3030, 2019.
6. Wu F, Sui Y, Wang Y, Xu T, Fan L and Zhu H: Long noncoding RNA SNHG7, a molecular sponge for microRNA-485, promotes the aggressive behavior of cervical cancer by regulating PAK4. Onco Targets Ther 13: 685-699, 2020.
7. Zhang S, Ding L, Gao F and Fan H: Long non-coding RNA DSCAM-AS1 upregulates USP47 expression through sponging miR-101-3p to accelerate osteosarcoma progression. Biochem Cell Biol 98: 600-611, 2020.
8. Wang R, Huang Z, Qian C, Wang M, Zheng Y, Jiang R and Yu C: LncRNA WEE2-AS1 promotes proliferation and inhibits apoptosis in triple negative breast cancer cells via regulating miR-32-5p/TOB1 axis. Biochem Biophys Res Commun 526: 1005-1012, 2020.
9. Fang Y, Zhang S, Yin J, Shen YX, Wang H, Chen XS and Tang H: LINC01535 promotes proliferation and inhibits apoptosis in esophageal squamous cell cancer by activating the JAK/STAT3 pathway. Eur Rev Med Pharmacol Sci 24: 3694-3700, 2020.
10. Yan Z, Zhang W, Xiong Y, Wang Y and Li Z: Long noncoding RNA FLVCR1-AS1 aggravates biological behaviors of glioma cells via targeting miR-4731-5p/E2F2 axis. Biochem Biophys Res Commun 521: 716-720, 2020.
11. Yan H, Li H, Silva MA, Guan Y, Yang L, Zhu L, Zhang Z, Li G and Ren C: LncRNA FLVCR1-AS1 mediates miR-513/YAP1 signaling to promote cell progression, migration, invasion and EMT process in ovarian cancer. J Exp Clin Cancer Res 38: 356, 2019.
12. Lin H, Shangguan Z, Zhu M, Bao L, Zhang Q and Pan S: lncRNA FLVCR1-AS1 silencing inhibits lung cancer cell proliferation, migration, and invasion by inhibiting the activity of the Wnt/β-catenin signaling pathway. J Cell Biochem 120: 10625-10632, 2019.
13. Luo H, Xu C, Le W, Ge B and Wang T: lncRNA CASC11 promotes cancer cell proliferation in bladder cancer through miRNA-150. J Cell Biochem 120: 13487-13493, 2019.
14. Peng CL, Zhao XJ, Wei CC and Wu JW: LncRNA HOTAIR promotes colon cancer development by down-regulating miRNA-34a. Eur Rev Med Pharmacol Sci 23: 5752-5761, 2019.
15. Lai XJ and Cheng HF: LncRNA colon cancer-associated transcript 1 (CCAT1) promotes proliferation and metastasis of ovarian cancer via miR-1290. Eur Rev Med Pharmacol Sci 22: 322-328, 2018.
16. Wang C, Huang Y, Zhang J and Fang Y: MiRNA-339-5p suppresses the malignant development of gastric cancer via targeting ALKBH1. Exp Mol Pathol 115: 104449, 2020.
17. Hong YG, Huang ZP, Liu QZ, E JF, Gao XH, Xin C, Zhang W, Li P and Hao LQ: MicroRNA-95-3p inhibits cell proliferation and metastasis in colorectal carcinoma by HDGF. Biomed J 43: 163-173, 2020.
18. Chen Y, Zhou J, Wu X, Huang J, Chen W, Liu D, Zhang J, Huang Y and Xue W: MiR-30a-3p inhibits renal cancer cell invasion and metastasis through targeting ATG12. Transl Androl Urol 9: 646-653, 2020.

19. Shang A, Zhou C, Bian G, Chen W, Lu W, Wang W and Li D: miR-381-3p restrains cervical cancer progression by downregulating FGF7. J Cell Biochem 120: 778-789, 2019.
20. Xie Y, Qi J, Zhu C, Zhao D and Liao G: MiR-381 functions as a tumor suppressor in gastric cancer by targeting ROCK2. Int J Clin Exp Pathol 12: 164-172, 2019.
21. Hu J, Wu X, Yang C, Rashid K, Ma C, Hu M, Ding Q and Jiang H: Anticancer effect of icaritin on prostate cancer via regulating miR-381-3p and its target gene UBE2C. Cancer Med 8: 7833-7845, 2019.
22. He X, Wei Y, Wang Y, Liu L, Wang W and Li N: MiR-381 functions as a tumor suppressor in colorectal cancer by targeting Twist1. Onco Targets Ther 9: 1231-1239, 2016.
23. 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.
24. Bao W, Cao F, Ni S, Yang J, Li H, Su Z and Zhao B: lncRNA FLVCR1-AS1 regulates cell proliferation, migration and invasion by sponging miR-485-5p in human cholangiocarcinoma. Oncol Lett 18: 2240-2247, 2019.
25. Liu Y, Guo G, Zhong Z, Sun L, Liao L, Wang X, Cao Q and Chen H: Long non-coding RNA FLVCR1-AS1 sponges miR-155 to promote the tumorigenesis of gastric cancer by targeting c-Myc. Am J Transl Res 11: 793-805, 2019.
26. Gao X, Zhao S, Yang X, Zang S and Yuan X: Long non-coding RNA FLVCR1-AS1 contributes to the proliferation and invasion of lung cancer by sponging miR-573 to upregulate the expression of E2F transcription factor 3. Biochem Biophys Res Commun 505: 931-938, 2018.
27. Zhang L, Cheng H, Yue Y, Li S, Zhang D and He R: TUG1 knockdown ameliorates atherosclerosis via up-regulating the expression of miR-133a target gene FGF1. Cardiovasc Pathol 33: 6-15, 2018.
28. Riaz F and Li D: Non-coding RNA associated competitive endogenous RNA regulatory network: Novel therapeutic approach in liver fibrosis. Curr Gene Ther 19: 305-317, 2019.
29. Wu D, Qin BY, Qi XG, Hong LL, Zhong HB and Huang JY: LncRNA AWPPH accelerates the progression of non-small cell lung cancer by sponging miRNA-204 to upregulate CDK6. Eur Rev Med Pharmacol Sci 24: 4281-4287, 2020.
30. Wang X, Chen K and Zhao Z: LncRNA OR3A4 regulated the growth of osteosarcoma cells by modulating the miR-1207-5p/G6PD Signaling. Onco Targets Ther 13: 3117-3128, 2020.
31. Meng Q, Wang L, Lv Y, Wu J and Shi W: Deletion of HNF1A-AS1 suppresses the malignant phenotypes of breast cancer cells in vitro and in vivo through targeting miRNA-20a-5p/TRIM32 axis. Cancer Biother Radiopharm: Apr 22, 2020 (Epub ahead of print). doi: 10.1089/cbr.2019.3168.
32. Zhang J, Wei Y, Min J, Wang Y, Yin L, Cao G and Shen H: Knockdown of RAP2A gene expression suppresses cisplatin resistance in gastric cancer cells. Oncol Lett 19: 350-358, 2020.
33. Zheng X, Zhao W, Ji P, Zhang K, Jin J, Feng M, Wang F, Zheng S and Wang X: High expression of Rap2A is associated with poor prognosis of patients with hepatocellular carcinoma. Int J Clin Exp Pathol 10: 9607-9613, 2017.
34. Wu JX, Du WQ, Wang XC, Wei LL, Huo FC, Pan YJ, Wu XJ and Pei DS: Rap2a serves as a potential prognostic indicator of renal cell carcinoma and promotes its migration and invasion through up-regulating p-Akt. Sci Rep 7: 6623, 2017.
35. Wu JX, Zhang DG, Zheng JN and Pei DS: Rap2a is a novel target gene of p53 and regulates cancer cell migration and invasion. Cell Signal 27: 1198-1207, 2015.