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RESEARCH

CircVIRMA enhances cell malignant behavior by governing the miR-452-5p/CREBRF pathway in cervical cancer

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Abstract

Our current study aimed to investigate the role and mechanism of circVIRMA in cervical cancer (CC) progression. CircVIRMA, microRNA-452-5p (miR-452-5p) and CREB3 regulatory factor (CREBRF) mRNA levels were examined in CC via quantitative real-time PCR (qRT-PCR). The protein level of CREBRF in CC was checked by Western blot. Cell Counting Kit-8 (CCK-8), colony formation, 5-Ethynyl-2′-deoxyuridine (EdU) staining, cell cycle, flow cytometry and transwell assays were conducted to estimate the effects of circVIRMA on malignant phenotypes of CC tumors. Western blot was used to measure related marker protein levels. The interaction between miR-452-5p and circVIRMA or CREBRF was predicted by bioinformatics analysis and verified by dual-luciferase reporter and RNA Immunoprecipitation (RIP) assays. Xenograft assay was used to assess the effect of circVIRMA on tumor growth in vivo. Immunohistochemistry (IHC) assay was performed to detect Ki-67 expression in tissues of mice. CircVIRMA and CREBRF levels were upregulated, while miR-452-5p was downregulated in CC tissues and cells. CircVIRMA silencing restrained CC cell proliferation, migration and invasion whereas induced apoptosis in vitro. In addition circVIRMA knockdown markedly attenuated xenograft tumor growth in vivo. circVIRMA was an efficient molecular sponge for miR-452-5p, and negatively regulated miR-452-5p expression. circVIRMA regulated CREBRF expression to modulate CC progression via miR-452-5p. MiR-452-5p downregulation reversed the effects of circVIRMA knockdown on CC progression. MiR-452-5p directly targeted CREBRF, and CREBRF overexpression partly restored the impact of miR-452-5p mimics on CC progression. circVIRMA mediated CC progression via regulating miR-452-5p/CREBRF axis, providing a novel therapeutic target for CC treatment.

Keywords Cervical cancer · CircVIRMA · MiR-452-5p · CREBRF

Highlights

1. CircVIRMA expression is increased in CC tissue specimens and cell lines.
2. CircVIRMA knockdown restrains the proliferation, migration and invasion abilities but induces the apoptosis of CC cells.
3. CircVIRMA/miR-452-5p/CREBRF axis is established in this study.
4. CircVIRMA accelerates the malignant potential of CC cells by sponging miR-452-5p to induce CREBRF expression.

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Introduction

Other than breast cancer, cervical cancer (CC) is the leading cause of cancer death in women (Sung et al. 2021). Currently, radiotherapy and surgery are the main strategies for the treatment of CC, although these treatments are not ideal for late-stage patients and recurrent patients (Abu-Rustum et al. 2020). CC brings huge economic loss and health burden to patients (Feng et al. 2019). At present, hysterectomy is becoming more and more popular, and is widely used as a treatment for early CC (Fader 2018). Thence, there is a pressing need for a detailed understanding of the mechanisms of CC progression to develop effective treatment plans.

Circular RNAs (circRNAs) are a naturally occurring family of non-coding RNAs, which can adjust gene level in eukaryotes (Jin et al. 2020). Strikingly, circRNA has become an important mediator involved in many human

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diseases and cancers (Han et al. 2018a). CircRNA has been determined to have carcinogenic or tumor suppressive potential as a biomarker (Chen and Huang 2018). CircRNA is a closed-loop RNA which derived from the reverse splicing of precursor mRNA (Kristensen et al. 2019).Compared with linear RNAs, circRNAs are not degraded by RNase R (Ma et al. 2021; Yin et al. 2020; Peng et al. 2020), indicating that they are more stable. Increasing evidences have indicated that circRNAs, as competitive endogenous RNAs (ceRNAs), participated in many cellular processes by inducing the downstream target genes expression through sponging miRNAs (Hansen et al. 2013; Yu et al. 2018). Increasing studies have affirmed that circRNAs play a significant role in CC. For instance, hsa_circ_0043280 negatively regulated the malignant process of CC by regulating the translation and stability of PAQR3 via miR-203a-3p (Zhang et al. 2021a). Moreover, mechanism studies have confirmed that circ_0102171 silencing down-regulated the expression of CREBRF through miR-4465 to inhibit cell growth and invasion, but enhance cell apoptosis (Tang et al. 2021). Additionally, hsa_circ_0004771 correlated with lymphovascular space invasion promoted migration and invasion by regulating the miR-629-3p/PTP4A1 axis and inducing the ERK1/2 pathway in CC (Li et al. 2020). However, the potential ability of circRNAs to subtly regulate the CC progression is still unknown, and further investigation is required. Previous studies have shown that circ_0084904 aggravated the development of cancer in CC by regulating the miR-802/MAL2 axis (Chen et al. 2022a). In the current study, we determined a new circRNA (circVIRMA) and verified its function in CC in vitro and in vivo. CircVIRMA (hsa_circ_0084904) is located at chr8:95,518,727–95,524,317 and derived from vir like m6A methyltransferase associated (VIRMA) gene. CircVIRMA was known to be up-regulated in the GSE102686 database (Jiao et al. 2020), but its potential role and mechanism are still unclear.

MiRNA is a size of non-coding RNA composed of 17–25 nucleotides, which participates in almost all biological processes such as cell invasion (Jiang et al. 2024). Many investigators believe that miRNA is expected to become one of the markers for clinical diagnosis of CC (Nahand et al. 2019). Previous studies had found that miRNAs were bound by circRNAs and affected the expression of downstream target genes involved in CC progression. For example, miR-299-3p (An et al. 2021), miR-203a-3p (Zhang et al. 2021a), miR-582-5p (Huang and Li 2021) and other miRNAs all participated in the process of CC by affecting their downstream. Moreover, circ-CCDC66 induced the expression of REXO1 via sponging miR-452-5p to accelerate CC progression (Zhang et al. 2021b). Whereas, it is not clear whether miR-452-5p participates in circVIRMA-mediated regulation on the occurrence and development of CC. CREB3 regulatory factor (CREBRF) was increased in CC and participated in the pernicious development of CC (Tang et al. 2021). But, whether CREBRF can be targeted by miR-452-5p to participate in the growth of CC is not yet clear.

In this research, we first confirmed that circVIRMA level was increased in CC tissue samples and cells than its matched control. Then it was proved that circVIRMA induced CREBRF expression to aggravate the CC process through sponging miR-452-5p. Our results reveal that circVIRMA may be a candidate target for CC therapy.

Materials and methods

Tissue selection

Sixty-three CC specimens and adjacent normal specimens were acquired from cases who did not receive preoperative chemoradiation or radiotherapy, and all patients had written an informed consent form prior to surgery at the Third People's Hospital of Zigong. In order to preserve the samples for a longer time, all case samples are frozen at − 80 °C. Human tissue specimens were utilized with the authorization of the Ethics Committee of the Third People's Hospital of Zigong.

Cell culture and transfection

The C-33A, HeLa and SiHa CC cell lines were derived from the American Type Culture Collection (Manassas, VA, USA). Normal cervical epithelial cells H8 was obtained from Tongpai (Shanghai, China). All cells were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (fetal bovine serum) (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were cultured in a constant temperature and humidity environment containing 5% CO_2.

The pCD5-ciR vector (GenScript, Nanjing, China) inserted full circVIRMA sequences was used for circVIRMA overexpression (circVIRMA), and the empty pCD5-ciR vector was acted as a matched control (circ-NC). Similarly, CREBRF was interposed into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA) to construct CREBRF overexpression vector (CREBRF), while the pure pcDNA3.1 vector served as a matching control (vector). Additionally, small interfering RNA (siRNA) against circVIRMA (si-circVIRMA #1, #2 and #3) and its matched control (si-NC), miR-452-5p overexpression/knockdown (miR-452-5p/anti-miR-452-5p) and their matched controls (NC/anti-NC) were derived from Genepharma (Shanghai, China). Specific short hairpin RNAs (shRNAs)-mediated circVIRMA silencing was used for in vivo assay. In short, Lipofectamine 2000 (Invitrogen) was used to transfect 30 nM oligonucleotides or 600 ng vectors into SiHa and HeLa cells.

Quantitative real-time PCR (qRT-PCR)

TRIzol® reagent (Invitrogen) was used to extract RNA. The specific operation was as follows: samples were added with TRIzol® reagent (Invitrogen), homogenized, and followed by subjected to a series of steps such as adding chloroform to obtain RNA. The RNA obtained first was synthesized using Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoorde, Belgium). Second, qRT-PCR was conducted applying SYBR Premix Ex Taq II (TaKaRa, Dalian, China). The above operations are carried out step by step according to the instructions. The specific primers used are presented in Table 1. The relative abundance of RNA was assessed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Cell cytoplasm/nucleus fraction isolation

Cytoplasmic/nuclear fractions were isolated using a nuclear/cytosol isolation kit (Cell Biolabs, Inc, USA). First, RNA was extracted from the cytoplasm or nucleus. Second, the relative expression levels of circVIRMA, VIRMA, nuclear control transcript (U6) and 18S rRNA were detected. 18S rRNA was used as an internal control for the cytoplasm and U6 for the nuclear fraction.

Treatment with RNase R or Actinomycin D

The expression of circVIRMA and VIRMA mRNA are discovered to mirror the molecular structure. RNA samples were added with 4 U/μg RNase R (Epicentre Technologies, Madison, WI, USA) for 40 min to digest linear RNAs. Subsequently, the expression of circVIRMA and VIRMA mRNA was detected by qRT-PCR. For the details of the actinomycin treatment, 2 mg/mL actinomycin D (Sigma-Aldrich) was added to the cell culture medium to detect the expression of circVIRMA and VIRMA mRNA at different time points.

Table 1 Specific primers in qRT-PCR assay

Name		Primers for PCR (5′-3′)
circVIRMA	Forward	CGCTAGCTAACTCTGAGAGCA
	Reverse	ACGTAAGGCAGTGGTAAGGC
VIRMA	Forward	GTAAGCAAACCAAGTGCCCC
	Reverse	CCAGCCTCTTAGCACCAGAC
miR-452-5p	Forward	GTATGAAACTGTTTGCAGAG
	Reverse	CTCAACTGGTGTCGTGGAG
CREBRF	Forward	GGAAGGTCCTGGGTCACTTG
	Reverse	TGGCTGTTCACCCAAGTTGT
GAPDH	Forward	GACAGTCAGCCGCATCTTCT
	Reverse	GCGCCCAATACGACCAAATC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
18S rRNA	Forward	AGAAACGGCTACCACATCCA
	Reverse	CCCTCCAATGGATCCTCGTT

Cell Counting Kit-8 (CCK-8) assay

The cell viability was gauged with a commercial CCK-8 kit (Beyotime, Shanghai, China) at different time points. The specific operations were as follows: Cells in the growth phase were collected by trypsinization and resuspended. An equal amount of cell suspension (100 μl) was added to each well to make 5000 cells per well. Note that 5 replicate wells were recommended for each treatment. CCK-8 solution (10 μl) was distributed to each well, and the incubation was continued for 2 h. Last, the absorbance was recorded at 450 nm.

Colony formation assay

The CC cells were resuspended and seeded in a 6-well plate, as far as possible to make the cells evenly dispersed. The cells were then cultured for another 2 weeks at 37℃ and 5% CO_2. Finally, the number of clones was detected, and the clone formation rate was counted.

5-Ethynyl-2′-deoxyuridine (EdU) assay

Cell proliferation was assayed with Commercial EdU Cell Proliferation Kit (Ribobio, Guangzhou, China) as required. In short, the CC cells were seeded on the culture plates to grow for 24 h, and then interacted with 10 μM EdU for 2 h. Hoechst 33,342 was used for nuclei staining. Observation of cells was performed with the aid of a fluorescence microscope (Olympus, Tokyo, Japan).

Cell cycle analyses

First, the cultured cells were harvested. Second, cells were trypsinized and fixed in ethanol, and finally these treated cells were filtered with a cell cycle analysis kit (Beyotime). Cells were analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). FlowJo 7.6 software (Tree Star, San Francisco, CA, USA) was used to analyze the relevant data.

Flow cytometry

Annexin V-FITC/PI apoptosis detection kit (Solarbio, Beijing, China) was used to disclose cell apoptosis. Cells transfected with the corresponding sequence were harvested after 48 h and then marked with Annexin V-FITC and PI. Flow cytometry was used to discover cell apoptosis.

Transwell assays

Transwell chambers (Corning, Tewksbury, MA, USA) coated without or with Matrigel matrix (Bedford, MA, USA) were used to detect cell migration. It should be explained that this detection device has two chambers, the upper chamber and the lower chamber, both need to add serum-free medium, the difference was that an additional 10% FBS needs to be added to the medium in the lower chamber. The first step was to prepare a cell suspension. Cells were then added to the upper chamber. After incubation for a period of time (24 h), the cells tried to move across the membrane to the bottom of the chamber, and the cells that penetrated the membrane were fixed with methanol (Beyotime), stained with 0.1% crystal violet (Beyotime), and counted under a microscope (Olympus).

Western blot

Tissues or transfected cells were treated with RIPA buffer (Sigma-Aldrich) to obtain protein. The protein was separated by SDS-PAGE gel and electro-transferred to PVDF membrane (Millipore, Billerica, MA, USA). Then the membrane was blocked using 5% nonfat milk and incubated with the primary antibodies including anti-Cyclin D1 (ab16663; 1:150; Abcam, Cambridge, UK), anti-CDK4 (ab108357; 1:5000), anti-Bax (ab182734; 1:1000), anti-caspase 3 (ab13847; 1:500), anti-Bcl-2 (ab182858; 1:2000), anti-MMP2 (ab97779; 1:2000), anti-MMP9 (ab76003; 1:10,000), anti-CREBRF (ab253202; 1:1000), anti-ki-67 (ab16667; 1:200), anti-GAPDH (ab181602; 1:10,000). Then, the goat-anti rabbit (ab205718; 1:10,000) was incubated with the membrane. Enhanced chemiluminescence reagent (Millipore) was used to display protein level.

Dual-luciferase reporter assay

It should be emphasized that the wild type was usually recorded as wt, and the mutant type was similarly recorded as mut. The circVIRMA-wt/mut or CREBRF-wt/mut vector was cloned into the pmirGLO dual-luciferase plasmid (Promega, Madison, WI, USA) to construct the respective recombinant plasmids. Then, miR-452-5p or NC was transfected with the above recombinant plasmids stained into CC cells. In order to check luciferase activity, the Dual Luciferase Assay Kit (Promega) was applied. Renilla luciferase was used for a control.

RNA immunoprecipitation (RIP)

Magna RNA immunoprecipitation kit (Millipore) was used to perform RIP experiments. SiHa and HeLa cells were lysed by adding RIP lysis buffer. Subsequently, the obtained extract was incubated with RIP buffer supplemented with anti-Argonaute2 (Ago2) antibody (Millipore) or control immunoglobulin G (IgG; Millipore) coupled magnetic beads (Invitrogen). Then, the RNA was detected by qRT-PCR.

Animal models

Twelve BALB/c nude mice were obtained from Vital River Laboratory Animal Technology Company (Beijing, China). 1×10^7 HeLa cells stably transfected with Lenti-sh-circVIRMA were injected subcutaneously into the flanks of six random nude mice. The other six were injected with the same number of HeLa cells stably transfected with Lenti-sh-NC at the same location. Tumor size was gauged with a vernier caliper every 5 days until the end of 30 days of culture, during which a total of 6 measurements were made. The volume of the subcutaneous tumor was computed using the Eq. $1/2 \times \text{length} \times \text{width} \times \text{width}$. Tumor samples were taken from mice injected subcutaneously for 30 days. Samples were weighed and stored for subsequent research and analysis.

Immunohistochemistry (IHC) assay

IHC staining was performed to assess ki-67 level in mouse subcutaneous xenografts. Briefly, tissue sections undergo a number of treatments such as antigen retrieval, endogenous peroxidase blocking, and nonspecific signal blocking. Subsequently, anti-ki-67 antibody (ab16667; 1:200; Abcam) was incubated with the sections overnight at 4 °C. Next, tissue sections were incubated with a biotinylated secondary antibody (SA00004-6; 1:100; ProteinTech Group, Chicago, IL, USA), and finally tissue sections were stained with 3, 3′-diaminobenzidine (DAB) substrate and counterstained with hematoxylin.

Statistical analysis

All experiments were carried out 3 times independently, and 3 biological replicates were set for each group. All results were carried out on GraphPad Prism 7.0 software (GraphPad, La Jolla, CA, USA) and exhibited as mean ± standard deviation (SD). Pearson correlation coefficient was used to analyze the linear correlations among circVIRMA, miR-452-5p and CREBRF. Student's *t*-test or analysis of variance was used to analyze data. Statistical significance of differences was indicated by $P < 0.05$.

Results

CircVIRMA level is elevated in CC tissues and cell lines

CircVIRMA was located on exon12-16 of VIRMA gene of chromosome 8, with a total length of 1346nt, and its circular junction site sequence was determined by Sanger sequencing (Fig. 1A). Compared with adjacent normal tissues ($n=63$), circVIRMA expression was observed to be notably elevated in CC tissues ($n=63$) (Fig. 1B). Besides, high circVIRMA expression was associated with more malignant phenotypes of CC patients (Table 2). Additionally, circVIRMA was up-regulated in CC cell lines (HeLa, SiHa and C-33A) compared with normal H8 cell line (Fig. 1C). With U6 and 18S rRNA as nuclear marker and cytoplasmic marker, circVIRMA was extremely enriched in the cytoplasm of CC cells (Fig. 1D). In general, RNase R could digest linear RNA but not circRNA. RNase R addition markedly degraded linear VIRMA mRNA but not circVIRMA (Fig. 1E), indicating that circVIRMA was a stable circRNA. Actinomycin D assay exhibited that circVIRMA had a longer half-life than linear VIRMA (Fig. 1F), indicating that circVIRMA transcript was more

Table 2 Correlative analysis of circVIRM level with clinicopathologic parameters of cervical cancer patients

Clinical feature		CircVIRM		
	n	High	Low	*P*-value
Age (years)				0.910
≥60	35	18	17	
<60	28	14	14	
Tumor differentiation				0.030*
Low	24	8	16	
High	39	24	15	
Tumor size (cm)				0.057
≥3	38	23	15	
<3	25	9	16	
TNM stage				0.016*
III+IV	36	23	13	
I+II	27	9	18	
Lymph node metastasis				0.033*
Negative	30	11	19	
Positive	33	21	12	

*$P<0.05$

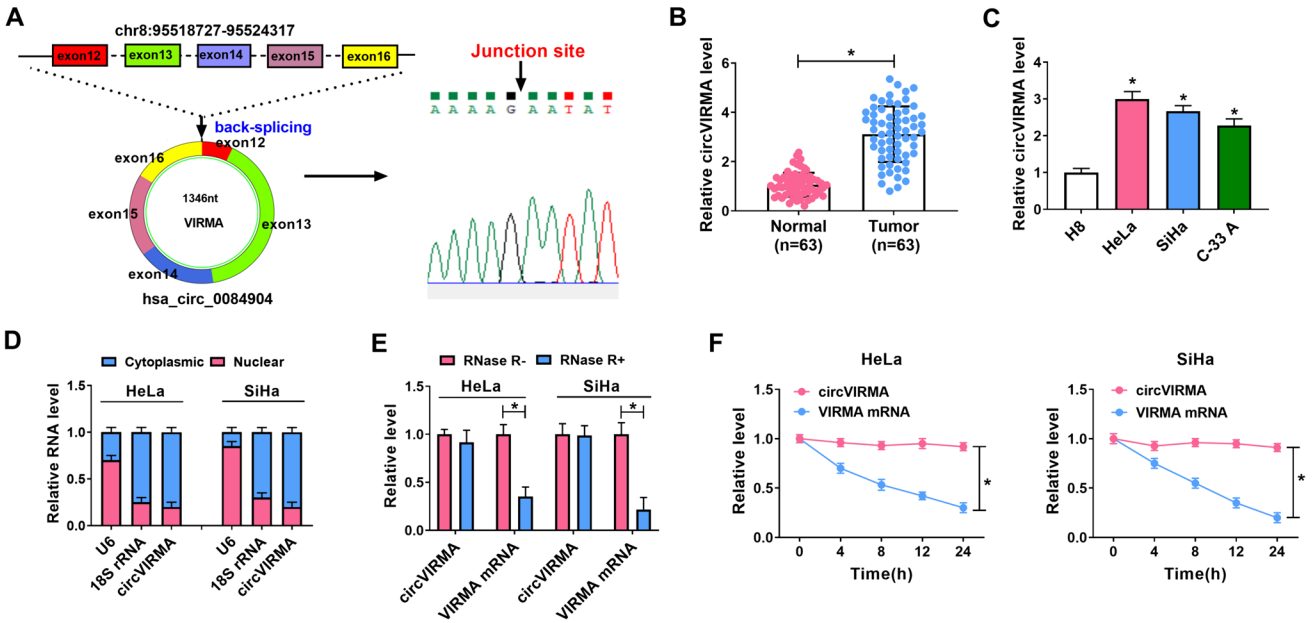

Fig. 1 CircVIRMA is expressed at high levels in CC tissues and cell lines. (**A**) The information of circVIRMA was presented and the determination of the circular junction site sequence of circVIRMA was achieved through Sanger sequencing. (**B**) The expression of circVIRMA in CC tissues and normal adjacent tissues was determined by qRT-PCR. (**C**) qRT-PCR was adopted to measure the expression of circVIRMA in all CC cell lines along with H8 cell line. (D) The nuclear and cytoplasmic separation experiment was used to explore the location of circVIRMA in CC cells. (**E**) After treatment of RNase R, circVIRMA and its linear transcript VIRMA mRNA were detected by qRT-PCR in CC cells. (**F**) The levels of circVIRMA and VIRMA mRNA in CC cells treated with Actinomycin D were examined using qRT-PCR at indicated time points (0, 4, 8, 12 and 24 h). *$P<0.05$

stable than the linear VIRMA mRNA transcript in CC cells. Overall, circVIRMA might regulate CC progression.

CircVIRMA exerts an oncogenic role in CC cells

To explore the function of circVIRMA in CC cells, we first suppressed the expression of circVIRMA in HeLa and SiHa cells by transfecting with siRNAs (si-circVIRMA#1, si-circVIRMA#2 and si-circVIRMA#3). CircVIRMA expression was markedly suppressed in three knockdown groups, with the highest transfection efficiencies in si-circVIRMA#1 group (Fig. 2A). CircVIRMA knockdown by siRNA obviously inhibited the proliferation of HeLa and SiHa cells, as indicated by the reduced OD value (Fig. 2B), colony formation ability (Fig. 2C), and the number of EdU-positive cells (Fig. 2D) in CC cells. The cell cycle analysis revealed a significantly increased number of cells in the G0-G1 phase and decreased number of cells in S phase after circVIRMA knockdown (Fig. 2E). The apoptotic rate of both CC cell lines was dramatically increased by circVIRMA silencing (Fig. 2F). Furthermore, reduced circVIRMA expression by siRNA inhibited cell migration and invasion in HeLa and

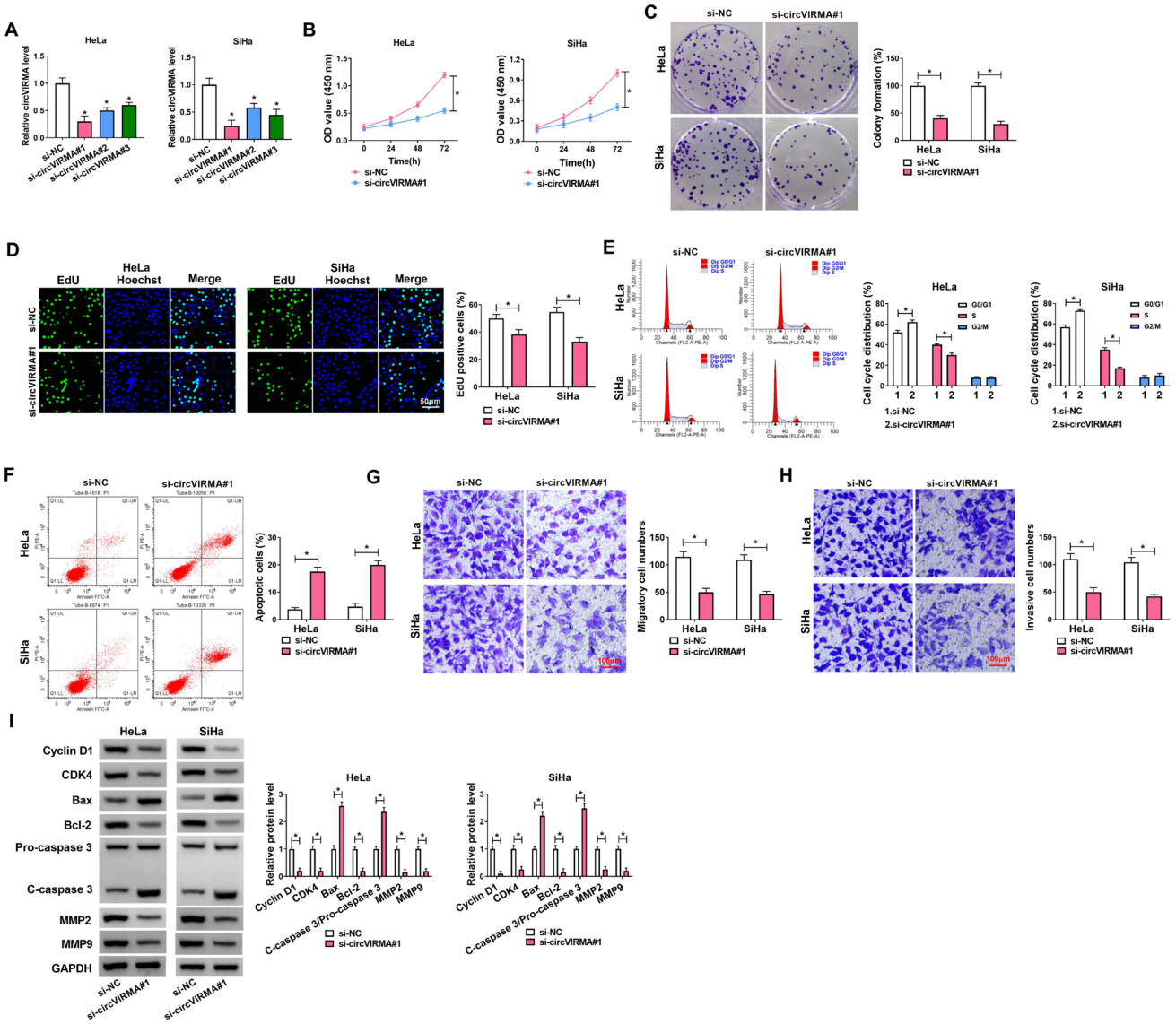

Fig. 2 CircVIRMA silencing attenuates cell proliferation, migration and invasion, and enhances apoptosis in CC cells. (**A**) CircVIRMA expression was measured by qRT-PCR in CC cells transfected with si-circVIRMA#1, si-circVIRMA#2, si-circVIRMA#3 or si-NC, respectively. (**B**–**I**) HeLa and SiHa cells were transfected with si-circVIRMA#1 or si-NC. Cell proliferation (**B**–**E**), apoptosis (F), migration (**G**) and invasion (**H**) were measured by CCK-8, colony formation, EdU staining, cell cycle assays, flow cytometry and transwell, respectively. (**I**) Western blot was performed to measure the protein expression of proliferation marker (Cyclin D1 and CDK4), apoptosis marker (Bax, Bcl-2 and caspase 3) and motility marker (MMP2 and MMP9). $*P < 0.05$

SiHa cells (Fig. 2G–H). CircVIRMA interference reduced the expression of Cyclin D1, CDK4, Bcl-2, MMP2 and MMP9 but up-regulated the level of Bax and cleaved caspase 3 (C-caspase 3)/Pro-caspase 3 (Fig. 2I). Overall, circVIRMA knockdown suppressed CC progression in vitro.

CircVIRMA acts as a sponge for miR-452-5p

We obtained miR-452-5p binding sites in circVIRMA from starbase (http://starbase.sysu.edu.cn/agoClipRNA.php?source=circRNA&flag=target&clade=mammal&genome=human&assembly=hg19&miRNA=all&clipNum=1&deNum=0&target=hsa_circ_0084904) and mutated the sites to construct circVIRMA-mut (Fig. 3A). Dual-luciferase reporter assay and RIP assay were used to detect the targeting relationship between miR-452-5p and circVIRMA. MiR-452-5p mimics evidently reduced the luciferase activity of wild-type reporter plasmid (circVIRMA-wt), but didn't affect the luciferase activity of mutant plasmid (circVIRMA-mut) in HeLa and SiHa cells (Fig. 3B). The RIP experiment further verified the binding between circVIRMA and miR-452-5p, because circVIRMA and miR-452-5p were enriched in Ago2 immunoprecipitates instead of IgG (Fig. 3C) in HeLa and SiHa cells. The expression of circVIRMA in HeLa and SiHa cells increased significantly after circVIRMA transfection (Fig. 3D). Furthermore, miR-452-5p level was obviously decreased by circVIRMA overexpression, but was notably elevated by circVIRMA knockdown (Fig. 3E). MiR-452-5p was significantly down-regulated in CC tumor tissues versus that in adjacent normal tissues (Fig. 3F). CircVIRMA was negatively correlated with the expression of miR-452-5p (Fig. 3G). MiR-452-5p expression was reduced in all CC cell lines when compared with H8

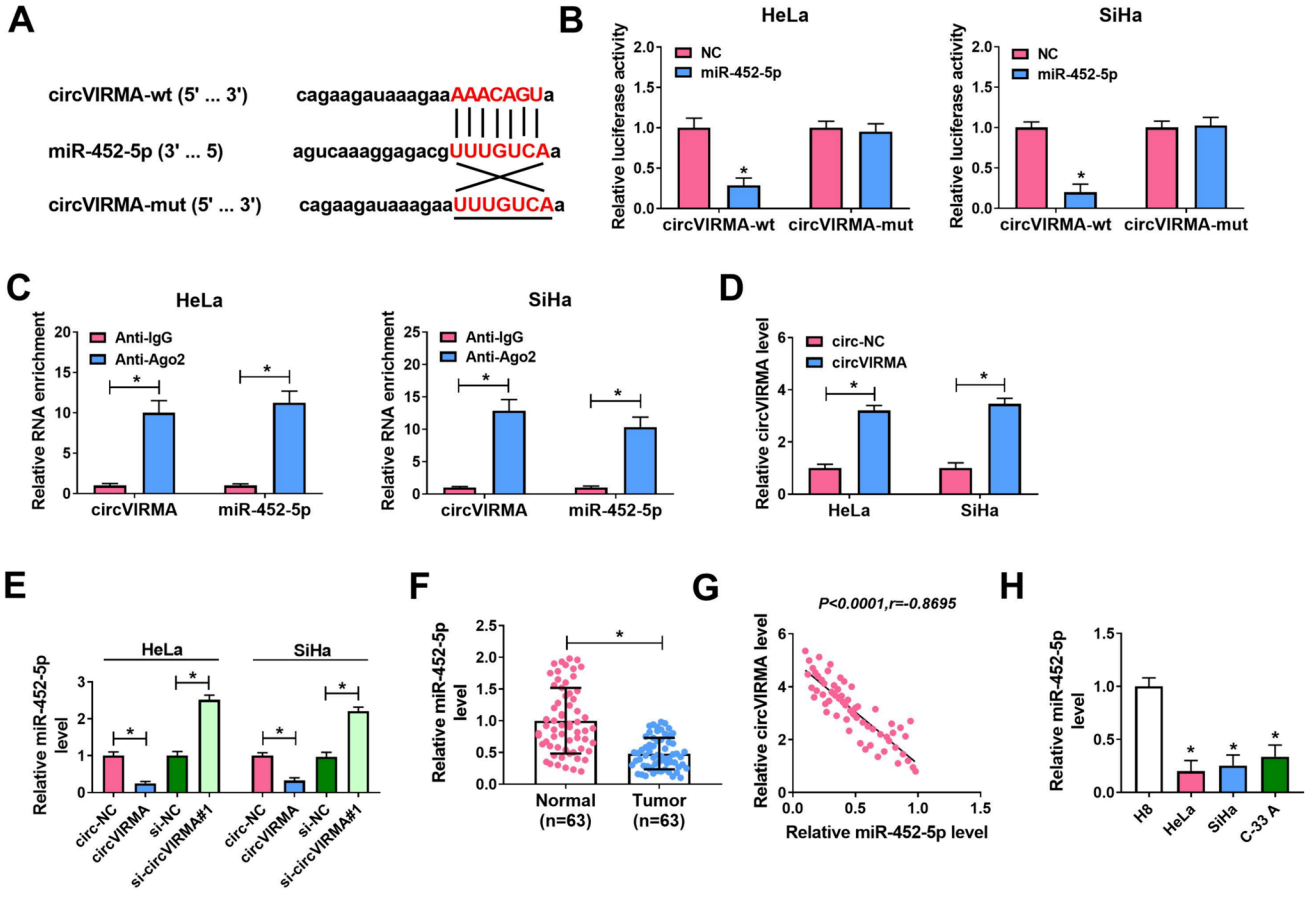

Fig. 3 CircVIRMA functions as a sponge of miR-452-5p. (**A**) The targets of circVIRMA were predicted using bioinformatic database starbase, and miR-452-5p was a candidate target of circVIRMA. (**B**–**C**) Dual-luciferase reporter assay and RIP assays in HeLa and SiHa cells were conducted to verify the binding between circVIRMA and miR-452-5p. (**D**) qRT-PCR was used to analyze the level of circVIRMA in HeLa and SiHa cells transfected with circ-NC or circVIRMA, respectively. (**E**) The effect of overexpression or knockdown of circVIRMA on the expression of miR-452-5p was examined by qRT-PCR. (**F**) The level of miR-452-5p was determined in CC samples ($n=63$) and matched normal samples ($n=63$) by qRT-PCR. (**G**) Linear correlation between the expression of circVIRMA and miR-452-5p was analyzed by Pearson correlation analysis. (H) qRT-PCR was adopted to measure the expression of miR-452-5p in H8 cells and CC cells. *$P<0.05$

cell line (Fig. 3H). Overall, circVIRMA directly interacted with miR-452-5p in CC cells.

MiR-452-5p knockdown rescues the effects of circVIRMA knockdown in CC cell progression

After knocking down miR-452-5p, the expression of miR-452-5p was down-regulated in CC cells (Fig. 4A). To investigate whether circVIRMA functioned by targeting miR-452-5p, restoration experiments were performed. si-circVIRMA elevated the level of miR-452-5p, while anti-miR-452-5p rescued this effect (Fig. 4B). The CCK-8 assay (Fig. 4C), colony formation assay (Fig. 4D) and EdU assay (Fig. 4E) confirmed that the inhibition of CC cell proliferation induced by circVIRMA silencing was largely attenuated by miR-452-5p knockdown. In addition, the effect of

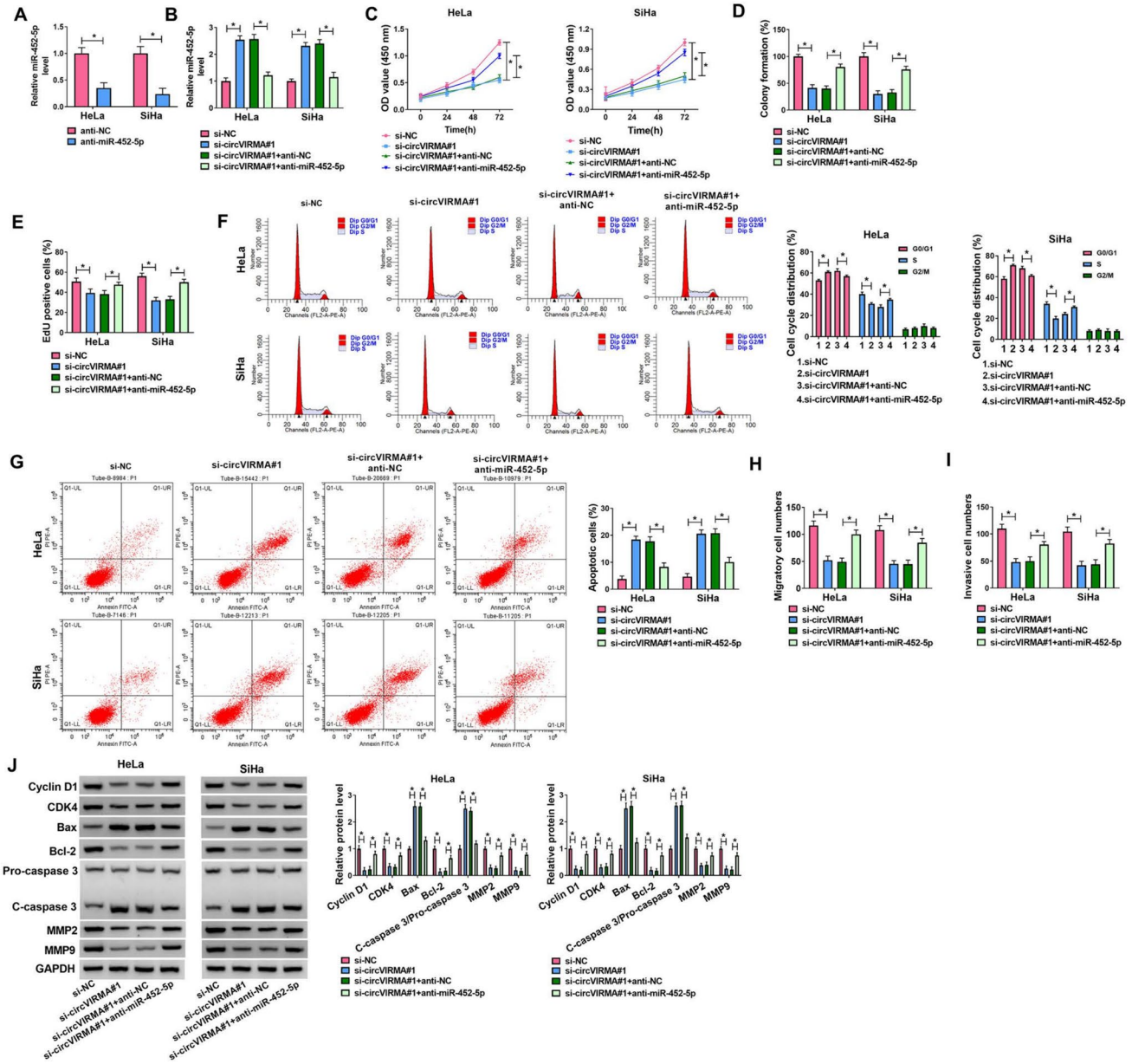

Fig. 4 CircVIRMA regulates the progression of CC by sponging miR-452-5p. (**A**) qRT-PCR was adopted to assess the silence efficiency of anti-miR-452-5p in CC cells. (**B**–**J**) CC cells were transfected with si-circVIRMA#1 or si-circVIRMA#1 + anti-miR-452-5p along with their matched controls. (**B**) The level of miR-452-5p was determined by qRT-PCR. (**C**–**F**) Cell proliferation was measured by CCK-8, colony formation, EdU staining and cell cycle assays. (**G**) Cell apoptosis was assessed by flow cytometry. (**H**–**I**) Cell migration and invasion were analyzed by transwell. (**J**) The protein levels related to cell proliferation, apoptosis and motility were determined by Western blot. $^{*}P < 0.05$

si-circVIRMA on the cell cycle progress was also weakened by anti-miR-452-5p (Fig. 4F). CircVIRMA silencing facilitated cell apoptosis, while anti-miR-452-5p restored this effect (Fig. 4G). Also, miR-452-5p knockdown reversed the effect of circVIRMA silencing on CC cell migration and invasion (Fig. 4H–I). Furthermore, the circVIRMA silencing-induced Bax and C-caspase 3/Pro-caspase 3 upregulation but Cyclin D1, CDK4, Bcl-2, MMP2 and MMP9 downregulation were alleviated by the addition of anti-miR-452-5p (Fig. 4J). Overall, the anti-tumor effects mediated by circVIRMA silencing were partly dependent on the upregulation of miR-452-5p.

MiR-452-5p binds to the 3′ UTR of CREBRF

To further reveal the molecular mechanism of miR-452-5p in CC, the downstream targets of miR-452-5p were sought by starbase. We examined CREBRF, which harbored the putative binding sites with miR-452-5p at its 3′UTR (Fig. 5A). Next, the luciferase activity of the cells co-transfected with miR-452-5p and CREBRF-wt was significantly reduced, while the luciferase activity of the CREBRF-mut group was almost unchanged (Fig. 5B). RIP assay further verified the binding between miR-452-5p and CREBRF (Fig. 5C). CREBRF expression was down-regulated by circVIRMA silencing, and this trend was attenuated by anti-miR-452-5p (Fig. 5D), indicating that circVIRMA positively regulated CREBRF expression by sponging miR-452-5p in CC cells. CREBRF mRNA and protein expression were both up-regulated in CC tissues compared with adjacent normal tissues (Fig. 5E and F). A positive correlation between the expression of circVIRMA and CREBRF mRNA in CC tissues was found (Fig. 5G), and there was a negative correlation between the expression of CREBRF mRNA and miR-452-5p (Fig. 5H). CREBRF protein level was elevated in CC cells versus that in normal H8 cells (Fig. 5I). Overall, CREBRF was a target of miR-452-5p in CC cells.

MiR-452-5p constrains the malignant behaviors of CC cells by regulating CREBRF

Transfection efficiencies of miR-452-5p mimics and CREBRF plasmid were high in CC cells (Fig. 6A and B). To explore if miR-452-5p exerted a tumor-suppressor role by

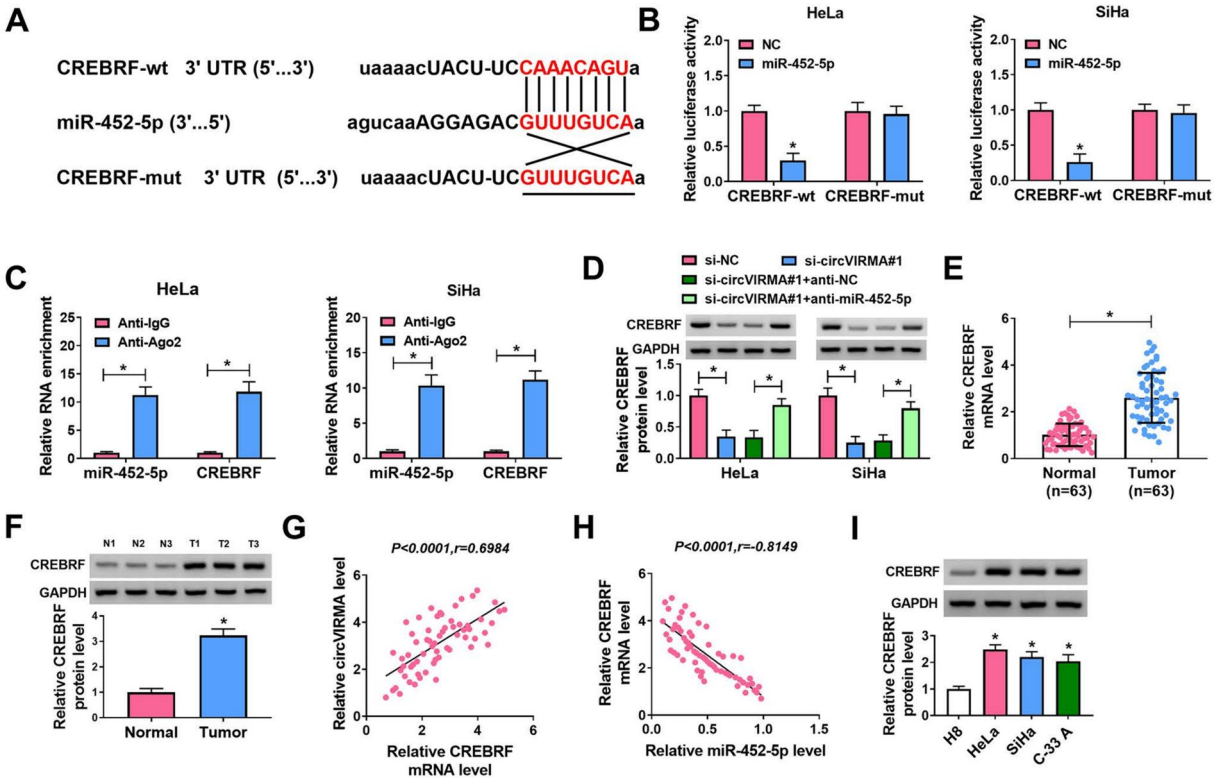

Fig. 5 MiR-452-5p binds to the 3′UTR of CREBRF. (**A**) The putative binding sites of miR-452-5p in CREBRF 3′UTR were shown. (**B** and **C**) Dual-luciferase reporter assay and RIP assay were performed to confirm the binding between miR-452-5p and CREBRF. (**D**) CREBRF protein level was examined in CC cells transfected with si-NC, si-circVIRMA#1, si-circ_0008035#1 + anti-NC, or si-circVIRMA#1 + anti-miR-452-5p, respectively, by Western blot. (**E** and **F**) The mRNA and protein levels of CREBRF were determined in CC tissues and adjacent normal tissues by qRT-PCR and Western blot. (**G** and **H**) Linear correlation between the expression of CREBRF mRNA and circVIRMA or miR-452-5p was evaluated. (I) Western blot was used to examine the protein level of CREBRF. $^{*}P < 0.05$

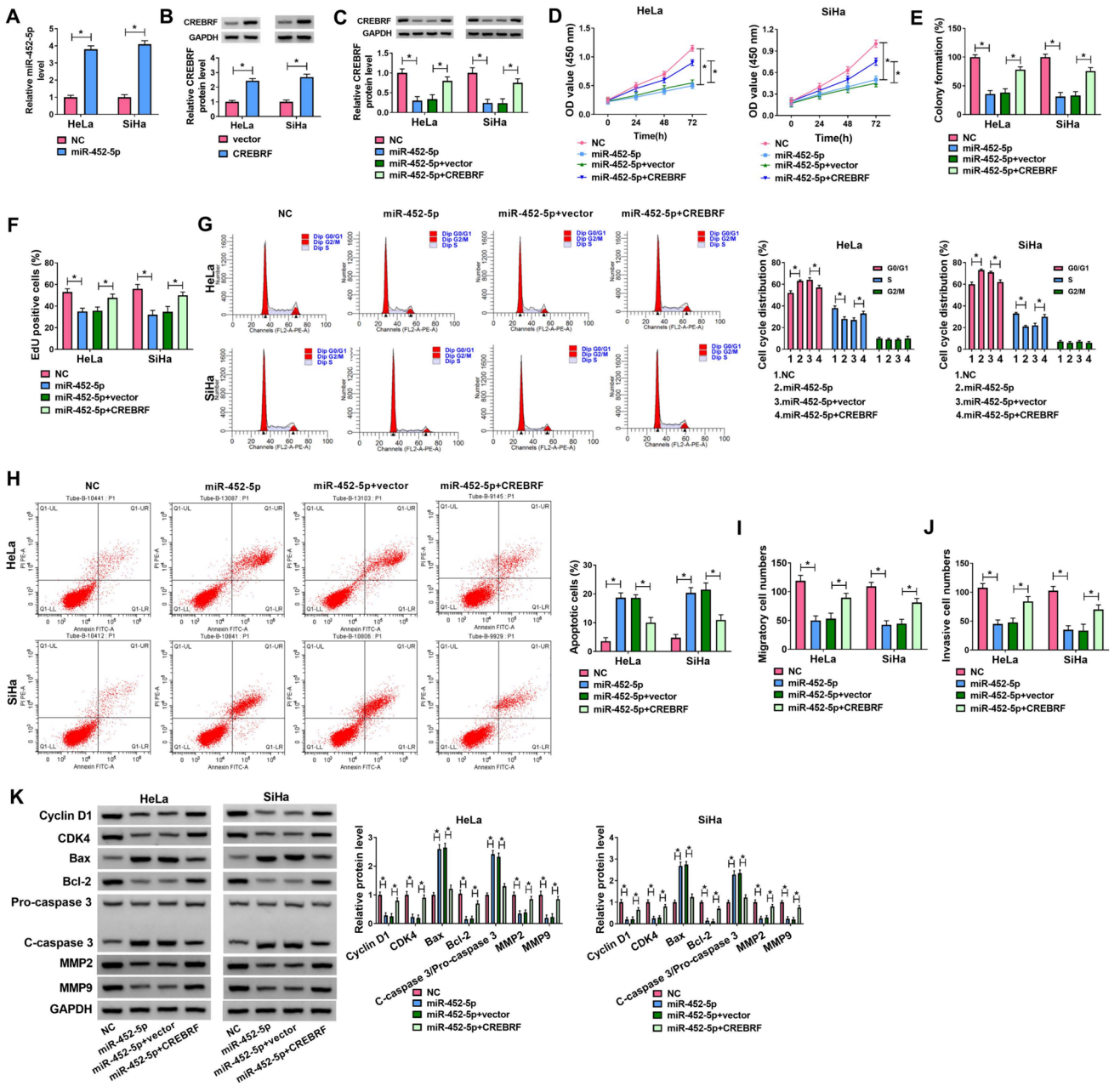

Fig. 6 MiR-452-5p inhibits the malignant behaviors of CC cells partly by targeting CREBRF. (**A**) Transfection efficiency of miR-452-5p was analyzed by qRT-PCR. (**B**) The protein level of CREBRF was detected using Western blot in cells transfected with vector or CREBRF. (**C**–**K**) CC cells were transfected with NC, miR-452-5p, miR-452-5p + vector or miR-452-5p + CREBRF, respectively. (**C**) CREBRF protein expression was determined by Western blot. Cell proliferation was assessed by CCK-8 assay (**D**), colony formation assay (**E**), EdU assay (**F**) and cell cycle assays (**G**). (H) Cell apoptosis was analyzed by flow cytometry. (**I**–**J**) Transwell was used to analyze cell migration and invasion. (**K**) Western blot was adopted to analyze protein expression. $^{*}P < 0.05$

targeting its target CREBRF, HeLa and SiHa cells were transfected with NC, miR-452-5p, miR-452-5p + vector or miR-452-5p + CREBRF, respectively. MiR-452-5p mimics repressed the level of CREBRF protein, and the inhibitory effect was reversed by CREBRF in CC cells (Fig. 6C). Moreover, CREBRF restored the inhibitory effect of miR-452-5p overexpression on the proliferation of CC cells (Fig. 6D–F). Overexpression of miR-452-5p significantly increased the number of cells in the G0-G1 phase and decreased the number of cells in the S phase, while these effects were reversed by CREBRF addition (Fig. 6G). MiR-452-5p markedly induced the apoptosis of CC cells and this tendency was abrogated by CREBRF upregulation (Fig. 6H). In addition, CREBRF overexpression reversed the effect of miR-452-5p

on CC cell migration and invasion (Fig. 4I–J). Additionally, miR-452-5p mimics decreased the expression of Cyclin D1, CDK4, Bcl-2, MMP2 and MMP9 whereas induced Bax and the cleavage of caspase 3 (Fig. 6K). Overall, miR-452-5p repressed CC progression by regulating CREBRF in vitro.

CircVIRMA knockdown significantly constrains xenograft tumor growth

To probe the functional effect of circVIRMA on CC development in vivo, the xenograft tumor model was established by injecting HeLa cells stably transfected with Lenti-sh-circVIRMA or Lenti-sh-NC into each group. After 30 days, we observed that all nude mice formed xenograft tumors at the inoculation site. Compared with the Lenti-sh-NC group, the tumor volume and weight were significantly reduced in Lenti-sh-circVIRMA group (Fig. 7A and B). CircVIRMA was markedly decreased, while miR-452-5p was evidently upregulated in Lenti-sh-circVIRMA group (Fig. 7C). Additionally, the protein level of CREBRF, Cyclin D1, CDK4, Bax, Bcl-2, caspase 3, MMP2 and MMP9 were significantly decreased, while Bax and the cleavage of caspase 3 level were exceptionally upregulated in Lenti-sh-circVIRMA group (Fig. 7D). Compared with the Lenti-sh-NC group, immunohistochemical staining showed that the number of ki-67-positive cells in xenograft tumors transfected with Lenti-sh-circVIRMA was reduced (Fig. 7E). Taken together, circVIRMA silencing curbed the growth of CC tumors in vivo.

Discussion

CC serves as one of the most common gynecologic cancers, and emerging evidence shows that circRNA plays an urgent role in the occurrence of CC (Chaichian et al. 2020). Although the role of circRNAs on CC had become the focus of attention, the subtle regulatory mechanism still needed

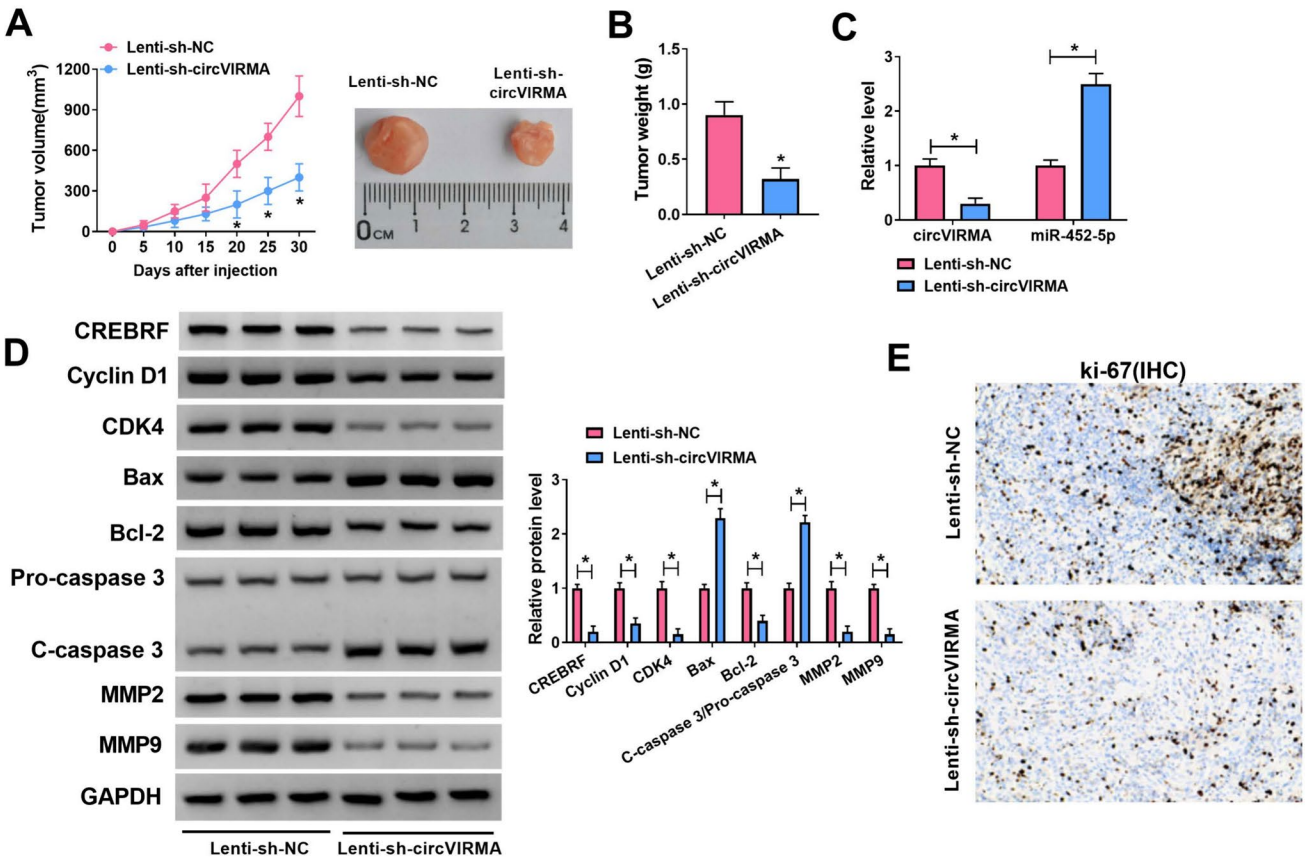

Fig. 7 CircVIRMA silencing significantly restrains xenograft tumor growth in vivo. HeLa cells stable transfected with Lenti-sh-circVIRMA or Lenti-sh-NC were injected into the nude mice to establish a xenograft model. Tumor growth was monitored every 5 days for 30 days. (**A** and **B**) Tumor volume and weight, (**C**) as well as circVIRMA, miR-452-5p were measured. (**C**) Western blot was used to analyze the levels of related proteins in xenograft tumor tissues including CREBRF, Cyclin D1, CDK4, Bax, Bcl-2, caspase 3, MMP2 and MMP9. (**E**) IHC assay was utilized to analyze the protein level of proliferation-related protein ki-67 in resected tumor tissues. $^{*}P < 0.05$

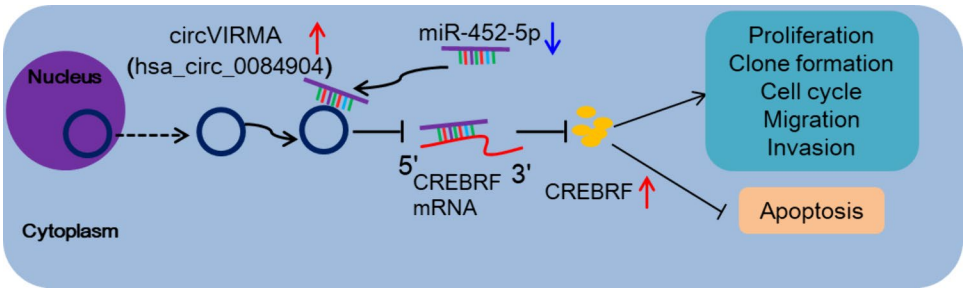

Fig. 8 CircVIRMA promotes the progression of CC by regulating the miR-485-5p/CREBRF axis

to be deeply explored. Here, we focus on the function of circVIRMA in CC. In the study, we found a circRNA (circVIRMA) that was abnormally up-regulated in a range of CC cells through GEO (GSE102686) analysis. At the same time, we verified that circVIRMA was elevated in the collected CC samples and cells compared with its matched controls, which aggravated the malignant progression of CC. Clone formation, CCK-8, EdU, cell cycle and flow cytometry experiments showed that circVIRMA knockdown curbed cell growth and promoted cell apoptosis in vitro. Additionally, experiments in vivo had also proved that circVIRMA silencing inhibited the growth of subcutaneously transplanted tumors. According to reports, circRNAs regulated the translation of target mRNAs and changed the expression of genes at the transcriptional level through sponging miRNAs (Zhong et al. 2018). More detailed regulatory network analysis on the role of circRNA-miRNA in CC progression was not discussed. In this study, we hoped to establish a theoretical premise for studies on CC therapy.

CircRNAs participated in the malignant process of CC with a variety of subtle regulatory mechanisms. For example, the combination of circZFR and SSBP1 induced p-Rb phosphorylation and E2F1 activation by accelerating the assembly of CDK2/cyclin E1 complex, which could promote cell proliferation (Zhou et al. 2021). Also, down-regulation of circAMOTL1 could inhibit the malignant phenotype of CC cells via miR-526b/SIK2 axis (Sun et al. 2020). Furthermore, both circAMOTL1 and AMOTL1 were elevated in CC samples than adjacent normal tissues, and circAMOTL1 promoted CC malignant development by controlling miR-485-5p/AMOTL1 (Ou et al. 2020). We searched that miR-485-5p had a base complementary site to circVIRMA by looking for the online prediction website. Therefore, we continued to explore the detailed regulation mechanism of circVIRMA.

In the current study, we found that circVIRMA was enriched in the cytoplasm and had a complementary base site to miR-485-5p. Previous studies have found that miR-485-5p negatively regulated tumor growth by regulating downstream target gene expression in most cancers including glioblastoma (Liu et al. 2021a), colorectal cancer (Zhang et al. 2021c), renal cell carcinoma (Liu et al. 2020) and esophageal squamous cell carcinoma (Liu et al. 2021b), etc. Circ_0074269 enhances TUFT1 expression and promotes DDP resistance in CC by binding to miR-485-5p (Chen et al. 2022). Consistent with the above research, we discovered that miR-485-5p was expressed at low levels in CC clinical samples/cells, and the addition of miR-485-5p inhibitor weakened the inhibitory effect of si-circVIRMA on CC cell development. The addition of miR-485-5p can restrain cell survival and induce apoptosis. Furthermore, miR-485-5p expression was negatively adjusted by circVIRMA.

In this study, CREBRF was predicted by the online website starbase as a downstream target of miR-485-5p. CREBRF was highly expressed in gastric cancer, and CREBRF knockdown retarded the activation of AKT signaling pathway, leading to cell proliferation arrest (Han et al. 2018). Our data displayed that CREBRF was elevated in CC clinical samples/cells. In addition, CREBRF level was adjusted by circVIRMA/miR-485-5p. Rescue experiments also proved that the addition of CREBRF could reduce the impact of miR-485-5p overexpression on CC cell progression. Otherwise, the linear correlations among circVIRMA, miR-485-5p, and CREBRF mRNA were evaluated, indicating that the relationship between miR-485-5p and the other two (circVIRMA/CREBRF mRNA) was negatively correlated, while the relationship between circVIRMA and CREBRF mRNA was positively correlated. These results illustrated that circVIRMA regulated the malignancy of CC cells by combining with miR-485-5p and inducing CREBRF expression.

It is difficult for any experimental research to be absolutely rigorous, and of course this research is no exception. We only proved that circVIRMA was involved in the growth of CC cells, but whether circVIRMA participated in CC cell glycolysis, drug resistance and the growth of HPV-infected CC cells was still unclear. In addition, the sample size of this study is not large enough. Despite its limitations, at least, we proved for the first time that circVIRMA aggravated the development of CC and provided a new idea for the treatment of CC in the future. Subsequently, a series of functional tests also proved that circVIRMA silencing could curb cell growth, and enhance cell apoptosis in CC cells. In short, the circVIRMA/miR-485-5p/CREBRF network intensified the progress of CC.

In summary, this study revealed that circVIRMA aggravated the progress of CC by targeting the miR-485-5p/CREBRF axis (Fig. 8), indicating that targeting circVIRMA may be a new idea to improve the therapeutic effect of CC. However, the detailed mechanism involved in circVIRMA/miR-485-5p/CREBRF axis in CC needs to be explored in the future.

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Authors contributions Conceptualization and Methodology: Jianjun Han and Kechao Xiang; Formal analysis and Data curation: Yun Wang, Xiangrui Chen and Chen Yang; Validation and Investigation: Chen Yang, Ailin Liang and Li Jia; Writing—original draft preparation and Writing—review and editing: Chengluo Hao, Jianjun Han and Kechao Xiang; Approval of final manuscript: all authors. The authors declare that all data were generated in-house and that no paper mill was used.

Data availability The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The present study was approved by the ethical review committee of the Third People's Hospital of Zigong. Written informed consent was obtained from all enrolled patients.

Consent for publication Patients agree to participate in this work.

Competing interests The authors declare no competing interests.

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