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Involvement of circular RNA SMARCA5/microRNA-620 axis in the regulation of cervical cancer cell proliferation, invasion and migration

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Abstract. – OBJECTIVE: Circular RNAs (circRNAs) have emerged as crucial regulators for tumor progression. However, the effects of circRNAs are not entirely clear in cervical cancer (CC). The objective of this study was to investigate the function and regulation mechanism of circular RNA SMARCA5 (circSMARCA5, also named hsa_circ_0001445) in cervical cancer.

PATIENTS AND METHODS: circSMARCA5 and miR-620 expressions were analyzed by qRT-PCR assay. The cell proliferation, cell cycle, migration, invasion abilities were measured by CCK-8 and colony formation, Flow cytometry, Wound-healing and transwell assays. In addition, the interaction between circSMARCA5 and miR-620 was accessed by luciferase reporter assay.

RESULTS: The results indicated that circSMARCA5 was downregulated in CC; overexpression of circSMARCA5 inhibited CC cell proliferation, migration and invasion, and induced cell cycle arrest. In addition, we found that circSMARCA5 could bind to miR-620 and significantly downregulated its expression. Furthermore, the results revealed that circSMARCA5 suppressed proliferation and invasion by miR-620 in CC.

CONCLUSIONS: We suggested that circSMARCA5/miR-620 regulatory axis is involved in the development of CC and may serve as a potential therapy target.

Key Words:
circSMARCA5, Cervical cancer, microRNA-620, Proliferation, Migration, Invasion.

Introduction

Cervical cancer (CC) is one of the most common female reproductive cancers worldwide[1]. The incidence of CC ranks second in female cancers, which is next to breast cancer[2]. The progress of CC is a very complex process, which is regulated by deregulation of gene functions at multiple stages[3]. However, the mechanism underlying the development of CC remains elusive. Circular RNAs (circRNAs) act as a class of endogenous noncoding RNAs, possess covalently closed loop structures which have no 5' to 3' polarity and polyadenylated tail[4]. Scholars[5-7] have determined that circRNAs could be produced through circularization of exons. Multiple studies have also shown that circRNAs serve as potential molecular markers of plentiful disease diagnosis and treatment, play essential roles in the development and progression of human diseases, especially cancers[8-10]. At present, circRNAs have become new molecular markers and therapeutic targets of various cancers[11,12]. However, the potential roles of circRNAs in CC are still not clear enough. In our study, we explored the expression levels of circSMARCA5 and mircoRNA-620 (miR-620) in CC tissue and cells, and the roles of circSMARCA5 on the proliferation, cycle, migration and invasion abilities in HeLa and C33A cells. In addition, we confirmed that circSMARCA5 served as miR-620 sponge and has significant effects in CC tumorigenesis.

Patients and Methods

Patients

CC tissues and adjacent non-tumor tissues were obtained following surgical resection from 30 patients treated at the First Affiliated Hospital of Bengbu Medical College. The current study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College. Written informed consent was obtained from all enrolled patients. None of the patients received radiotherapy or chemotherapy prior to surgical resection in the study. The samples were washed with sterile phosphate-buffered saline (PBS; Cat#

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311-010-CL; Wisent, Inc., Montreal, Canada), and immediately conserved at -80°C until use.

Cell Culture

Human CC cell lines (HeLa, Caski, SiHa and C33A) and 293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were incubated in Dulbecco modified Eagle medium (DMEM, Cat#: 11960-044, Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Cat. No. P4333, Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS; Cat. No. 10438-026, Gibco, Rockville, MD, USA). All cells were incubated at 37°C in an atmosphere of 5% CO_2.

Plasmid Construction

The complete sequence of human circSMARCA5 gene was synthesized and inserted into a pcDNA3.1 (+) vector (GenePharma, Shanghai, China) at with 5'-BamHI and 3'-NotI restriction sites. 293T cells (5×10^6 in every 10-cm culture dish) were co-transfection with packaging vectors in Opti-MEM (Cat. # 319-047, Gibco, Rockville, MD, USA) and incubated for 48 hrs at 37°C. Subsequently, the virus was purified by using ultracentrifugation (25000 ×g for 2 hrs, 4°C). HeLa and C33A cells (5×10^5 cells) were seeded into 6-well plates and incubated overnight at 37°C. Next day, HeLa and C33A cells were transfected with circSMARCA5 lentiviruses and vector lentiviruses by using 8 μg/ml ploybrene (Sigma-Aldrich, St. Louis, MO, USA). The stable cells were screened by using geneticin (G418) (0.8 mg/ml, Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

MiRNA Transfection

MiR-620 mimics and miRNA controls (scramble) were obtained from Shanghai GeneChem Co., Ltd. (Shanghai, China). The sequences are as follows: miR-620 mimics, 5'-AUG GAG AUA GAU AUA GAA AU-3'; miRNA controls, 5'-UUC UCC GAA CGU GUC ACG UTT-3'. HeLa and C33A cells (1×10^5 cells) were seeded in 6-well plates and transfected with miR-620 mimics and scramble, respectively, using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from the 30 pair CC tissues and the transfected HeLa and C33A cells by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher, No. K1622, Waltham, MA, USA) according to the manufacturer's instructions. The expression levels of circSMARCA5 and miR-620 were detected by qRT-PCR assay with a SYBR Green PCR Master Mix kit (TaKaRa Biotechnology Co., Ltd., Otsu, Shiga, Japan) on an ABI-Prism 7300 system (Applied Biosystems; Inc., Foster City, CA, USA). GAPDH or U6 was used to internal reference. miR-620 expression was normalized to U6, and the expression level of circSMARCA5 was normalized to GAPDH. The results were counted using $2^{-\Delta\Delta Ct}$ calculation[13]. GAPDH primers were: F, 5'-TGT TCG TCA TGG GTG TGA AC-3', R, 5'-ATG GCA TGG ACT GTG GTC AT -3'; circSMARCA5 primers were: F, 5'-CAA GAT GGG CGA AAG TTC ACT-3', R, 5'-TCT CAC CTT CTT TGC ACC TCT-3'; U6 primers were: F, 5'-CTC GCT TCG GCA GCA CA-3', R, 5'-AAC GCT TCA CGA ATT TGC GT-3'; miR-620 primers were: F, 5'-ACA CTC CAG CTG GGA TGG AGA TAG ATA T-3', R, 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG ATT TCT AT-3'

Polymerase Chain Reaction (PCR)

PCR assay was performed by using 2×Taq PCR MasterMix (Tiangen Biotech Co., Ltd, Beijing, China). The reaction system (50 μL) was as follows: 2×MasterMix (25 μL), each primer (3 μL), cDNA template (1μg), and water. The reaction process was as follows: 94°C for 5 mins, followed by 94°C for 30 s, 58°C for 30 s, 72°C for 1 min for 30 cycles, with a final step for 5 min at 72°C. PCR products were measured by using electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer on a 1.0% agarose gel. The sequences of divergent primers of circSMARCA5 were: F, 5'-CAA GAT GGG CGA AAG TTC ACT-3', R, 5'-TCT CAC CTT CTT TGC ACC TCT-3'; the sequences of convergent primers were forward 5'-GGA GGC TTG TGG ATC AGA ATC-3', reverse 5'-TCT CAC CTT CTT TGC ACC TCT-3'.

Dual-Luciferase Reporter Assay

The binding site of circSMARCA5-3'UTR and miR-620 was carried out by using Target-Scan (http://www.targetscan.org/vert_71/) and miRanda (http://34.236.212.39/microrna/home.do). Subsequently, the wild-type and mutant circSMAR-

CA5-3'UTR were synthesized and cloned into the pGL3-promoter vector (Promega Corporation, Madison, WI, USA; Cat. No. E1751). HeLa and C33A cells (5×10^4 cells/well) were seeded in 24 well plates and co-transfected with miR-620 or scramble, and wild-type or mutated 3′-UTR circSMARCA5 for 48 hrs, respectively. The Renilla luciferase vector pRL SV50 (Promega Corp., Madison, WI, USA) was internal reference. The results were detected by a Dual Luciferase Reporter Assay system (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Cell Proliferation Analysis

For CCK-8 assay, the treated HeLa and C33A cells (2×10^3 cells/well) were seeded in 96-well plates and at 37°C. CCK-8 reagent (Cat. No. CK04) was added at 1, 2, 3, and 4 days, respectively. The absorbance was detected by using a microplate reader (Polarstar Optima, BMG Labtech.) at 450 nm. For colony formation assay, the treated HeLa and C33A cells (500 cells/well) were seeded into 6-well plate. After 2 week, the cells were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich, Cat. 16005) and stained with 0.5% crystal violet (Sigma-Aldrich, Cat. # C-3886). The number of colonies was counted to access the colony formation ability.

Cell Cycle Analysis

Cell cycle distribution was performed with propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO, USA) staining at indicated times. The treated HeLa and C33A cells were harvested and fixed, and then incubated with propidium iodide (PI) staining solution (10 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) including 0.1% Triton X-100 and RNase (100 mg/l) for 30 mins in the dark. The results were measured on a FACSCalibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Wound-Healing Assay

The treated HeLa and C33A cells (2×10^4 cells/well) were plated in 6-well plates with serum-free medium and incubated at 37°C. A straight scratch was made by a 10 μl plastic pipette tip. Images of these scratches were obtained at 0 and 48 h under a microscope.

Invasion Assays

The treated HeLa and C33A cells (5×10^4 cells/well) were seeded in the top of Matrigel-coated invasion chambers (BD Biosciences, Franklin Lakes, NJ, USA) with serum-free media. The complete medium with 10% fetal bovine serum (FBS) was added into the lower chambers. After 24, the non-invading cells were removed, the invasive cells were fixed by 95% ethanol, and stained by 0.1% crystal violet solution (Cat# C-3886, Sigma-Aldrich, St. Louis, MO, USA). The images were captured under a microscopy with a magnification of ×100.

Bioinformatics Method

The miRNA targets predicted by computer-aided algorithms were obtained from Circular RNA interactome (https://circinteractome.nia.nih.gov/index.html).

Statistical Analysis

Experimental data are displayed as the mean ± standard deviation (SD). All analyses were performed using variance (ANOVA) using SPSS software (version 12.0; SPSS, Inc., Chicago, IL, USA); p-value of <0.05 was considered to indicate a statistical significance difference.

Results

CircSMARCA5 is Downregulated in CC

CircSMARCA5 was located in chr4:144464661-144465125, and from the circular exon 15 and 16 of SMARCA5. The genomic length was 464 bp, the spliced sequence length was 269 bp. The best transcript of circSMARCA5 was NM_003601 (Figure 1 A). To verify that exons 15 and 16 of the SMARCA5 gene formed an endogenous circRNA, we designed the divergent and convergent primers that generated the back-splicing or linear products from SMARCA5 gene. The results indicated that divergent primers can produce circSMARCA5 in cDNA, and not in gDNA; and the convergent primers can amplify the PCR products from linear SMARCA5 mRNA in HeLa cells and CC tissue (Figure 1 B). Those data proved the existence of circSMARCA5 in CC. In addition, we found that circSMARCA5 expression was significantly downregulated in CC tissues compared with paired normal tumor tissues (n=30, $p<0.05$, Figure 1 C); circSMARCA5 expression was also lower in stages I-II and stages III-IV than that in cervical intraepithelial neoplasias (CIN) ($p< 0.05$, Figure 1 D). Furthermore, we performed qRT-PCR assay to evaluate the circSMARCA5 expression in CC cells (HeLa, Caski, SiHa and C33A), and the results revealed that circSMARCA5 ex-

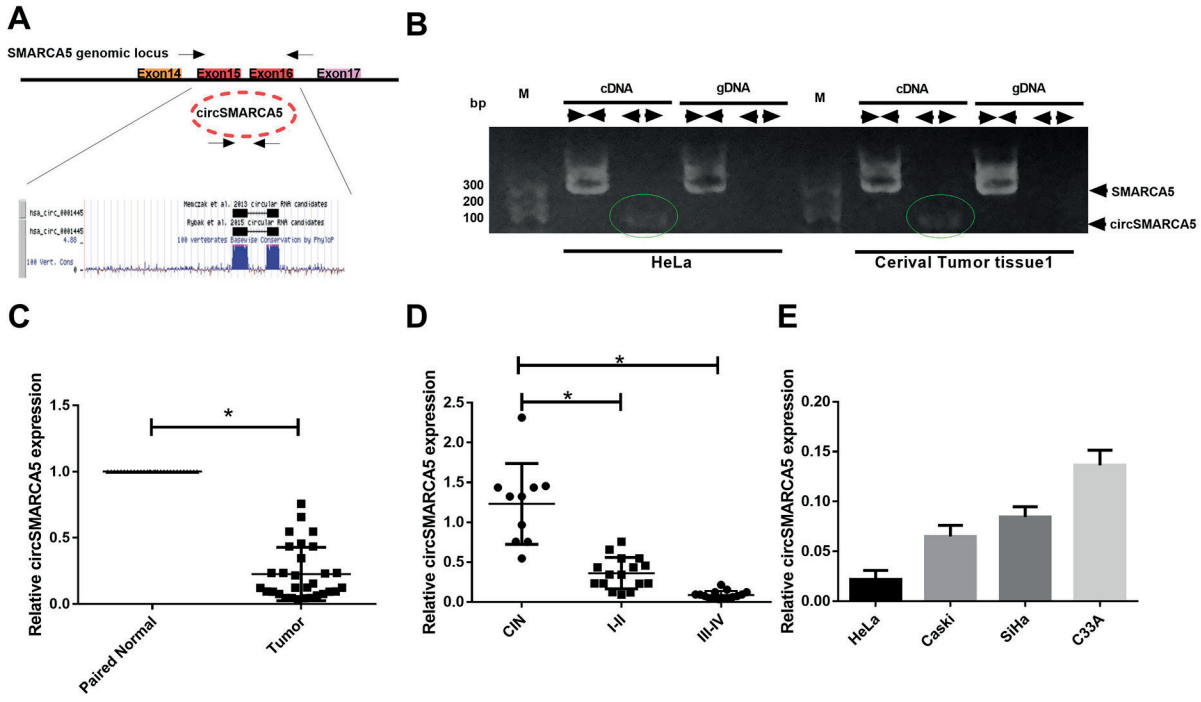

Figure 1. CircSMARCA5 is downregulated in CC. ***(A)*** Schematic diagram of the annotated genomic region of SMARCA5 and the formation of circular exon 15 and 16 (circSMARCA5). ***(B)*** Divergent and convergent primers were designed to generate the back-splicing or linear products. The total RNAs were obtained from HeLa cells and CC tissue. The results were detected by PCR assay. ***(C)*** CircSMARCA5 expression was analyzed by qRT-PCR assay in CC tissues and paired normal tumor tissues (n=30, *p<0.05). ***(D)*** qRT-PCR assay was used to evaluate the circSMARCA5 expression in cervical intraepithelial neoplasias (CIN), stages I-II, and stages III-IV (*p<0.05). ***(E)*** CircSMARCA5 expression was measured by qRT-PCR assay in HeLa, Caski, SiHa and C33A cells (*p<0.05).

pression was lowly expressed in HeLa cells, and highly expressed in C33A cells (Figure 1 E).

CircSMARCA5 Suppresses CC Cell Tumorigenesis

In order to investigate the possible roles of circSMARCA5 in CC, circSMARCA5 plasmid and vector were constructed, and transfected into HeLa and C33A cells, qRT-PCR assay was used to detect the transfection effects. The results manifested that circSMARCA5 expression was significantly increased in circSMARCA5 group compared with vector group (p<0.05, Figure 2A). Therefore, we demonstrated that there was an efficient transfection for circSMARCA5. Whereafter, flow cytometry detection was performed to access the cell cycle distribution of HeLa and C33A cells, and we found that the number of cells was significantly increased in G1 phase, and decreased in S phase in circSMARCA5 group compared with vector group (p<0.05, Figure 2B). In addition, the data from CCK-8 and colony formation assays indicated that overexpression of circSMARCA5 dramatically inhibited the proliferation and colony-forming abilities of HeLa and C33A cells (p<0.05, Figure 2C-2D). Furthermore, we found that the invasion ability was significantly decreased in HeLa and C33A cells transfected with circSMARCA5 compared with vector (p<0.05, Figure 2E-2F). The results from wound healing assay also demonstrated that the migration ability was significantly decreased in circSMARCA5 group compared with vector group (p<0.05, Figure 2G-2H). Therefore, we suggested that circSMARCA5 inhibited the migration and invasion abilities of CC cells.

CircSMARCA5 Interacts with miR-620

Recently, researches[14-16] have indicated that circRNAs affect the effects of miRNAs on their target genes during cell proliferation, apoptosis, migration and invasion through functioning as competitive endogenous RNAs (ceRNAs). In our study, the potential target miRNAs were predicted by TargetScan, miRanda (Figure 3A); there were 17 miRNAs, which included the binding sites of

circSMARCA5, and miR-620 ranked relatively higher than others. To verify the results of the predicted ceRNA, we designed and constructed the mutated 3′-UTR circSMARCA5 according to the binding sites of circSMARCA5 and the predicted miR-620 (Figure 3B). HeLa and C33A cells were co-transfected with miR-620 or scramble, and wild-type or mutated 3′-UTR circSMARCA5, respectively. The results from the luciferase reporter assays demonstrated that the luciferase activity of circSMARCA5 was significantly reduced in HeLa and C33A cells transfected wild-type 3′-UTR circSMARCA5 and miR-620 compared with scramble group (p<0.05, Figure 3C). There was no significant effect on the luciferase activity when HeLa and C33A cells were co-transfected with mutated 3′-UTR circSMARCA5 and miR-620 (Figure 3D). Moreover, our results revealed that overexpression of circSMARCA5 significantly downregulated miR-620 expression in HeLa

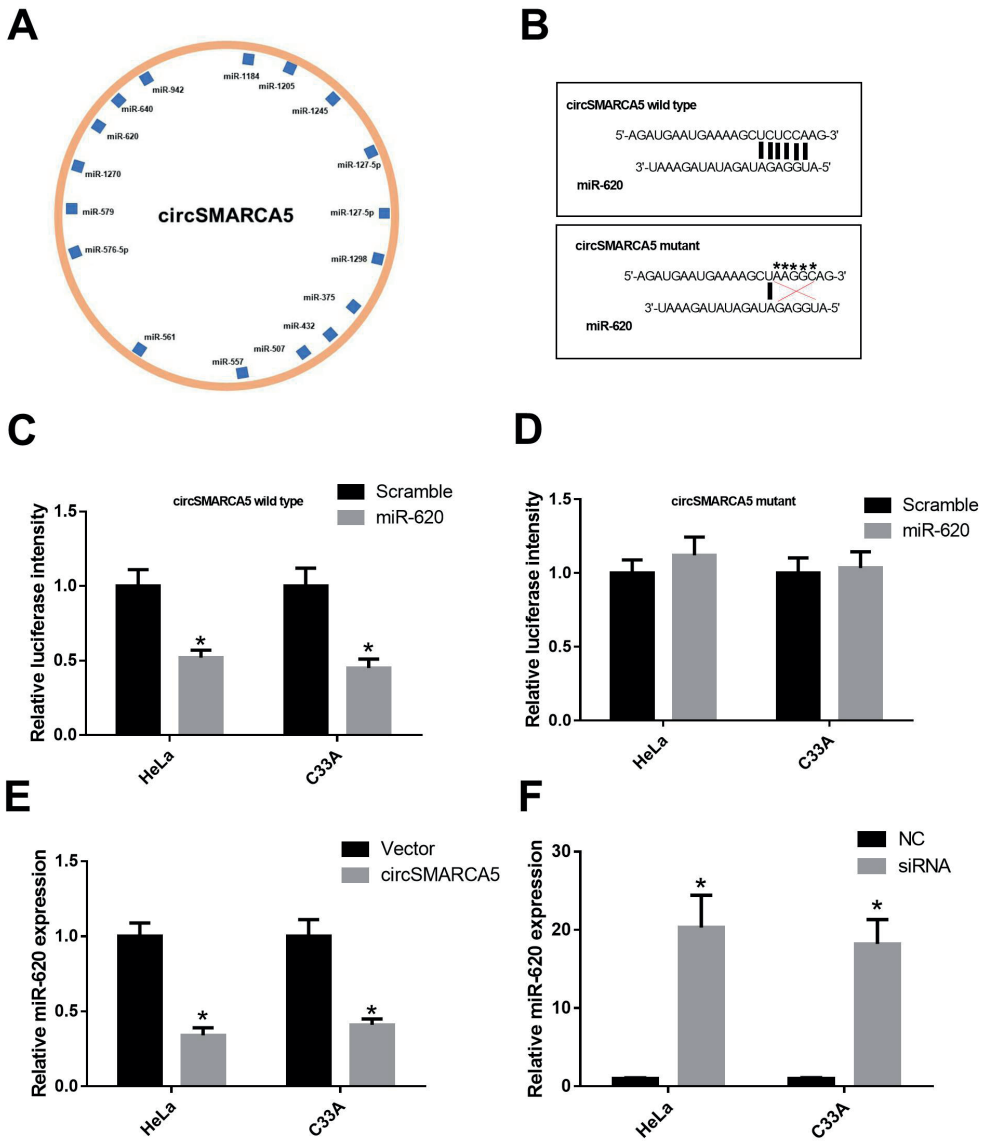

Figure 2. CircSMARCA5 suppresses CC cell tumorigenesis. HeLa and C33A cells were transfected with circSMARCA5 plasmid or Vector, respectively. (**A**) CircSMARCA5 expression was measured by qRT-PCR assay in treated HeLa and C33A cells (*p<0.05). ***(B)*** The cell cycle was detected by flow cytometry in treated HeLa and C33A cells, and the values of G1, S, and G2 phase were shown in the bar graphs (*p<0.05). ***(C-D)*** CCK-8 and colony formation assays were performed to measure the proliferation abilities of HeLa and C33A cells (*p<0.05). HeLa and C33A cells were transfected with circSMARCA5 plasmid or Vector, respectively. ***(E-F)*** Transwell assay was used to detect cell invasion ability (*p<0.05). ***(G-H)*** Wound Healing assay was performed to detect cell migration capacity (*p< 0.05).

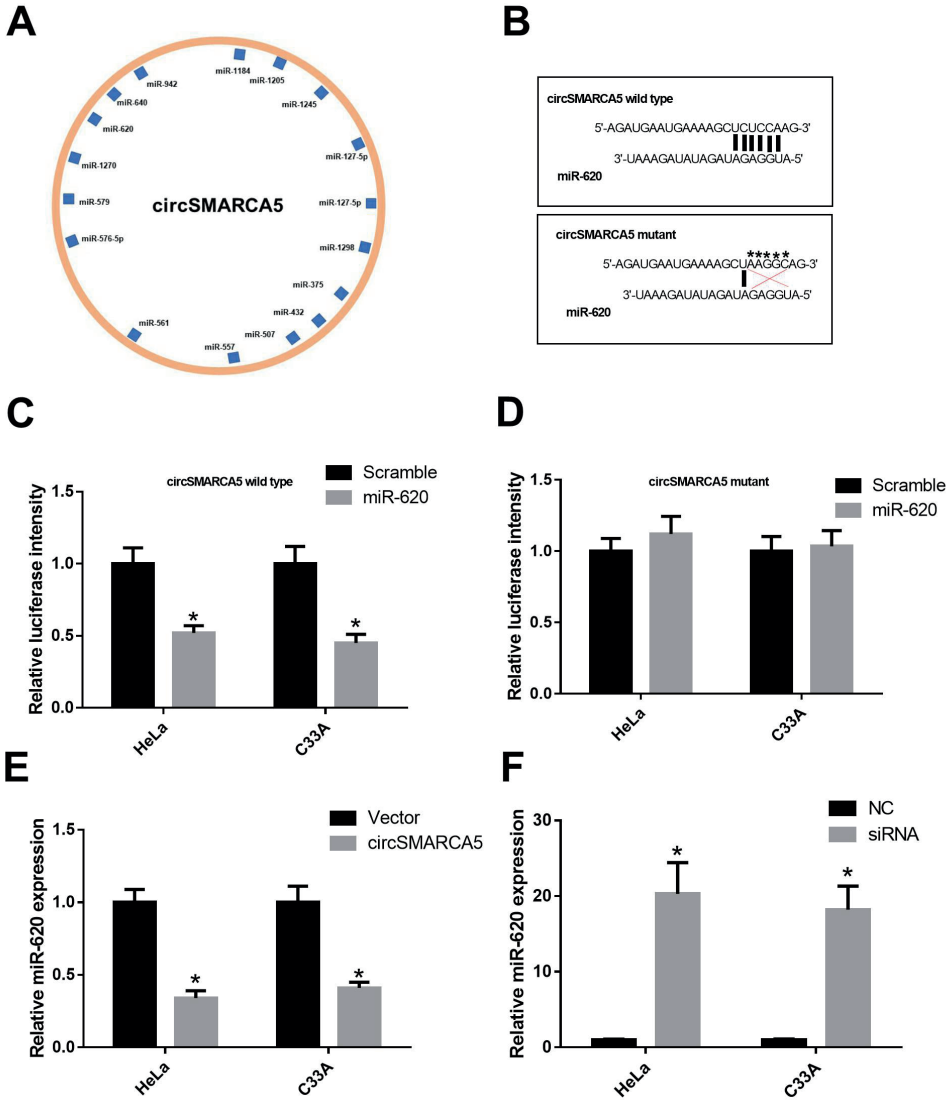

Figure 3. CircSMARCA5 interacts with miR-620. ***(A)*** Schematic diagram of circSMARCA5 and potential target miRNAs. (**B**) The binding sites of the wild-type and mutated 3′-UTR circSMARCA5 and miR-620. ***(C-D)*** HeLa and C33A cells were co-transfected with miR-620 or scramble, and wild-type or mutated 3′-UTR circSMARCA5, respectively. The luciferase intensity of circSMARCA5 was measured by Dual-Luciferase Reporter Assay (*p<0.05). ***(E)*** HeLa and C33A cells were transfected with circSMARCA5 or vector, and miR-620 expression was analyzed by qRT-PCR assay (*p<0.05). ***(F)*** HeLa and C33A cells were transfected with circSMARCA5 siRNAs or negative control (NC), and miR-620 expression was detected by qRT-PCR assay (*p< 0.05).

and C33A cells (p<0.05, Figure 3E) and silence of circSMARCA5 by siRNAs significantly upregulated miR-620 expression in HeLa and C33A cells (p<0.05, Figure 3F). All of these results indicated that circSMARCA5 could bind to miR-620 and inhibit its expression.

CircSMARCA5 Inhibits Proliferation and Invasion by miR-620 in CC

To further explore the expression and regulatory mechanism of miR-620 in CC, qRT-PCR assay was performed to analyze the expression level of miR-620 in CC tissues, and the results indicated that miR-620 expression was significantly upregulated in CC tissues relative to paired normal tumor tissues (p<0.05, Figure 4A). Besides, we found that there was a negative correlation between circSMARCA5 and miR-620 in 30 CC tissues (p<0.05, r^2=0.1653, Figure 4B). Subsequently, we evaluated the biological functions of circSMARCA5 on CC by regulating miR-620 in vitro. HeLa cells were transfected with vector,

circSMARCA5 plasmid, circSMARCA5 and miR-620, respectively. The results from colony formation assay showed that circSMARCA5 dramatically promoted the colony forming ability of HeLa cells, and miR-620 then attenuated this promotion mediated by circSMARCA5 ($p<0.05$, Figure 4C). The results from Transwell assay proved that circSMARCA5 markedly accelerated the invasion ability of HeLa cells, and miR-620 then weakened this acceleration mediated by circSMARCA5 ($p<0.05$, Figure 4D). Therefore, we suggested that circSMARCA5 from the circular exon 15 and 16 of SMARCA5 inhibits CC cell proliferation, migration and invasion by acting as miR-620 sponge (Figure 4E).

Discussion

Researchers[17-23] have proved that circRNAs may be closely connected with the development of various cancers. Hsa_circ_0001445 (circSMARCA5) is from the circular exon 15 and 16 of SMARCA5. Previous studies have shown that circSMARCA5

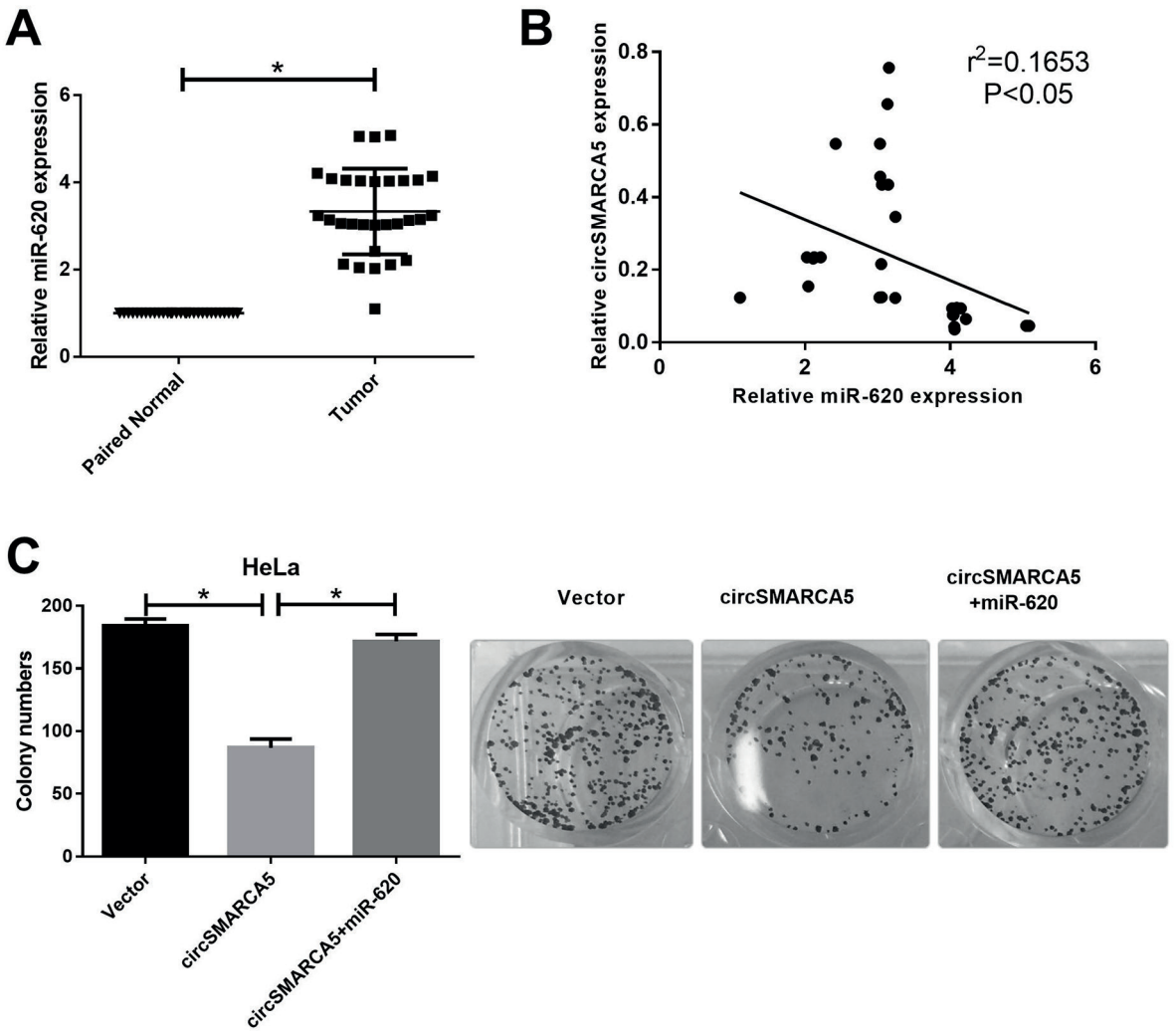

Figure 4. CircSMARCA5 inhibits proliferation and invasion by miR-620 in CC. ***(A)*** MiR-620 expression was assessed by qRT-PCR assay in CC tissues and paired normal tumor tissues (n=30, *$p< 0.05$). ***(B)*** Pearson's correlation was used to analyze the correlation between circSMARCA5 and miR-620 expression in 30 CC tissues ($p<0.05$, $r^2=0.1653$). ***(C-D)*** HeLa cells were transfected with vector, circSMARCA5 plasmid, circSMARCA5 and miR-620, respectively. The proliferation and invasion capacities were measured by colony formation and transwell assays (*$p< 0.05$). ***(E)*** The diagrammatic drawing was shown about the antitumor effects of circSMARCA5 in CC by miR-620.

Figure continued

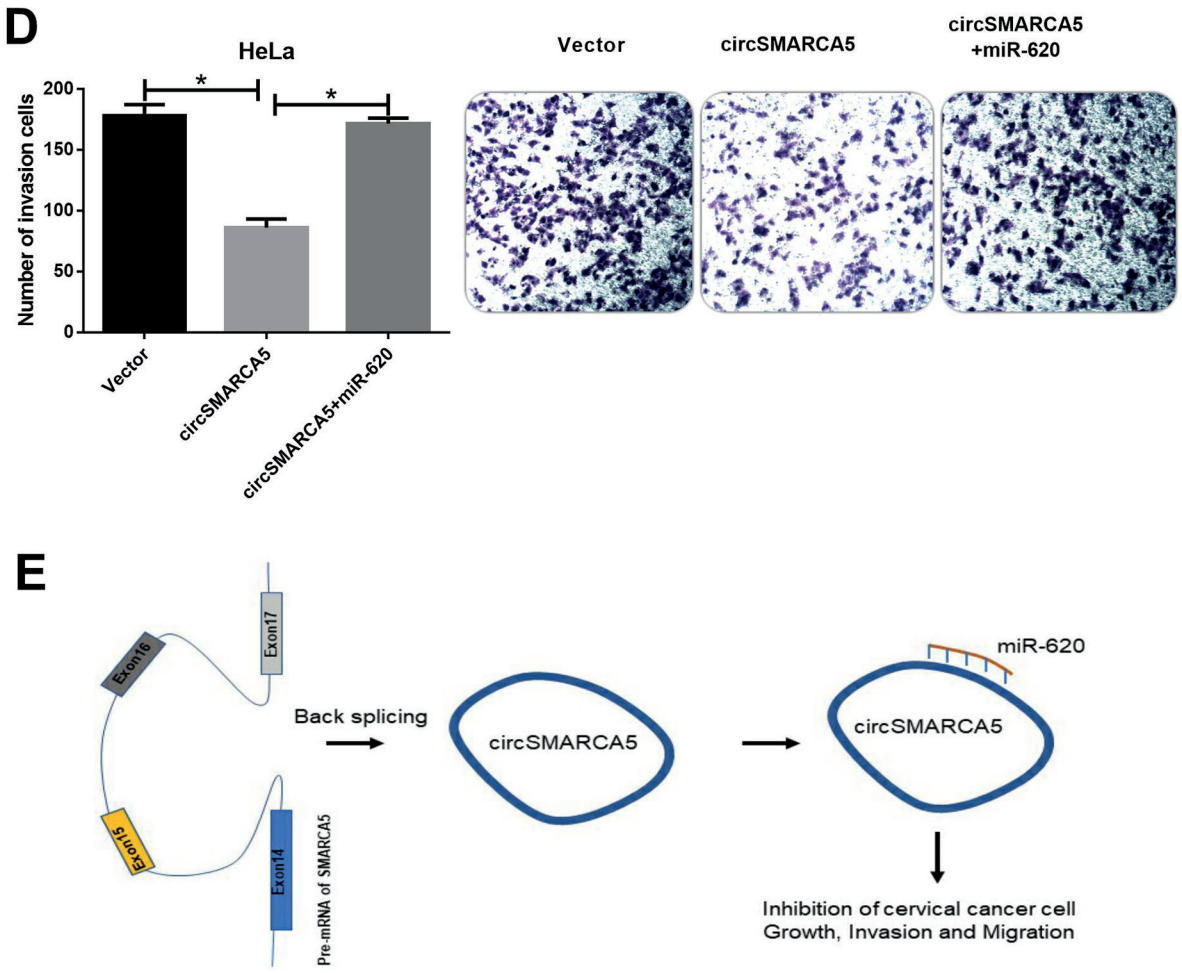

Figure 4. *Continued.* **CircSMARCA5 inhibits proliferation and invasion by miR-620 in CC.** ***(A)*** MiR-620 expression was assessed by qRT-PCR assay in CC tissues and paired normal tumor tissues (n=30, *$p< 0.05$). ***(B)*** Pearson's correlation was used to analyze the correlation between circSMARCA5 and miR-620 expression in 30 CC tissues ($p<0.05$, $r^2=0.1653$). ***(C-D)*** HeLa cells were transfected with vector, circSMARCA5 plasmid, circSMARCA5 and miR-620, respectively. The proliferation and invasion capacities were measured by colony formation and transwell assays (*$p< 0.05$). ***(E)*** The diagrammatic drawing was shown about the antitumor effects of circSMARCA5 in CC by miR-620.

plays essential roles in the development and progression of cancers. For instance, circSMARCA5 (hsa_circ_0001445), may act as a diagnostic biomarker, and suppress the proliferation, migration and metastasis of hepatocellular carcinoma[24]. The upregulation of circSMARCA5 promoted prostate cancer cell proliferation, and inhibited cell apoptosis[25]; circSMARCA5 inhibited the migration ability of glioblastoma multiform cells by SRSF1/SRSF3/PTB axis[26]; circSMARCA5, acted as a diagnostic biomarker, affected the proliferation and migration of hepatocellular carcinoma[21]. However, the roles of circSMARCA5 in CC development are unknown. We found that the expression level of the circSMARCA5 was significantly lower in CC tissues than paired normal tumor tissues. CircSMARCA5 expression was also related to CC I- IV stages. Consistent with our results in CC tissues, we also found that CC cells (HeLa, Caski, SiHa and C33A) revealed lower expression level of circSMARCA5 compared to cervical intraepithelial neoplasias (CIN). Moreover, our findings indicated that overexpression of circSMARCA5 inhibited CC cell proliferation, and induced cell cycle arrest; simultaneously, overexpressed circSMARCA5 suppressed CC cell migration and invasion. Therefore, circSMARCA5 may be a potential biomarker and provide an effective ther-

apeutic target for CC. MiRNAs, a class of small non-coding RNAs with 19-24 nucleotides, are differentially expressed in various cancers[27-29]. Researches[28-31] increasingly suggested that miRNAs can act as oncogenes or tumor suppressors, and can affect the proliferation, cycle, apoptosis, metastasis, angiogenesis and inflammation of tumors. Investigations also have indicated that miR-620 functions as a cancerogen in numerous cancers. For example, miR-620 participated in the biological process of lung adenocarcinoma[32]; miR-620 promoted tumor radio resistance through 15-hydroxyprostaglandin dehydrogenase (HPGD)[33]; miR-620 was upregulated in colorectal cancer[34]. However, the detailed understandings for the mechanisms of miR-620 still need to be investigate to further improve the diagnosis, prevention and treatment of CC. In our study, our results indicated that miR-620 was upregulated in CC tissues compared with paired normal tumor tissues; circSMARCA5 was negatively correlated to miR-620 expression in CC. In addition, we found that overexpression of circSMARCA5 significantly decreased miR-620 expression and that silence of circSMARCA5 significantly increased miR-620 expression in CC.

Conclusions

We observed the functional mechanism of circSMARCA5-integrated miR-620 in CC. Firstly, we found that circSMARCA5 inhibited CC cell proliferation, migration and invasion, and induced cell cycle arrest. Secondly, we revealed that circSMARCA5 was downregulated and miR-620 was upregulated in CC tissues; there was a negative correlation between circSMARCA5 and miR-620 in CC tissues. Furthermore, circSMARCA5 was indicated to be involved in the processes of proliferation and invasion in CC through miR-620. Therefore, circSMARCA5 might sponge to miR-620 to modulate the biological processes of CC.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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