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THERAPY

Circular RNA circSLC26A4 Accelerates Cervical Cancer Progression via miR-1287-5p/HOXA7 Axis

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Circular RNAs (circRNAs) are group of noncoding RNAs derived from back-splicing events. Accumulating evidence certifies the critical roles of circRNAs in human tumorigenesis. However, the role and biogenesis of circRNAs in cervical cancer are still unclear. Here, a novel identified circRNA, circSLC26A4, was found to be upregulated in cervical cancer tissue and cells. Clinically, the high expression of circSLC26A4 was related to the poor survival of cervical cancer patients. Functionally, cellular experiments indicated that circSLC26A4 knockdown repressed the proliferation, invasion, and tumor growth _in vitro_ and _in vivo_. Furthermore, circSLC26A4 acted as the sponge of miR-1287-5p; moreover, miR-1287-5p targeted the 3′ UTR of HOXA7 mRNA. Mechanistically, RNA binding protein (RBP) quaking (QKI) was identified to interact with the QKI response elements (QREs) in SLC26A4 gene introns, thereby promoting circSLC26A4 biogenesis. In conclusion, these findings demonstrate that circSLC26A4 facilitates cervical cancer progression through the QKI/ circSLC26A4/miR-1287-5p/HOXA7 axis, which might bring novel therapeutic strategies for cervical cancer.

INTRODUCTION

Cervical cancer acts as one of the most common gynecologic cancers, accounting for a large proportion of cancer-related mortality worldwide.[1,2] An increasing number of cervical cancer cases occur every year in developed and developing countries.[3] The difficulty for cervical cancer treatment is the inaccuracy of early diagnosis and the ambiguity for therapeutic targets.[4] Although great advancements have been acquired on cervical cancer chemotherapy, radiotherapy, and surgical therapies, the lethality for cervical cancer is still rigorous.

Circular RNAs (circRNAs) are a class of RNA transcripts generated by back splicing, involving into the transcriptional and post-transcriptional gene regulation.[5,6] Existing reports find that circRNAs derived from the exons are mainly distributed in the cytoplasm,[7,8] and circRNAs derived from the introns are primarily distributed in the nucleus.[9,10] circRNAs regulates a series of pathophysiological processes of human cancers. For example, circAKT3 is higher in cisplatin-resistant gastric cancer tissues and cells, which are significantly associated with aggressive characteristics and acts as an

independent risk factor for gastric cancer.[11] Recent research has disclosed that circRNAs could code polypeptide. For example, circ-ZNF609 could be translated to be a protein in a splicing-dependent/cap-independent manner modulated by stress conditions.[12]

In this research, we identified a new circRNA in cervical cancer. The novel circRNA is derived from the exons (exons 4, 5, and 6) of the SLC26A4 gene, thereby being named as circSLC26A4 (circBase: hsa_circ_0132980; genomic location: chr7:107312582-107315554). Moreover, we found that circSLC26A4 could be catalyzed by the RNA binding protein (RBP) quaking (QKI). A functional investigation found that circSLC26A4 could accelerate cervical cancer progression through targeting the miR-1287-5p/HOXA7 axis. Our finding confirms that circSLC26A4 functions as an oncogene in cervical cancer tumorigenesis, acting as a microRNA (miRNA) sponge. Besides, the data provide a novel insight for the biogenesis of circRNA in cervical cancer.

RESULTS

circSLC26A4 Is Overexpressed in Cervical Cancer Tissue and Cells

Our anterior sequencing analysis found that circSLC26A4 was originated from exons 4, 5, and 6 of the SLC26A4 gene. The genomic location of circSLC26A4 is chr7:107312582-107315554, and the circBase ID (http://www.circbase.org/) is hsa_circ_0132980 (Figure 1A). Sanger sequencing revealed that the back-splicing junction site of exon 6 toward exon 4 was detected in the RNA extraction, suggesting that there was indeed circular construction (Figure 1B).

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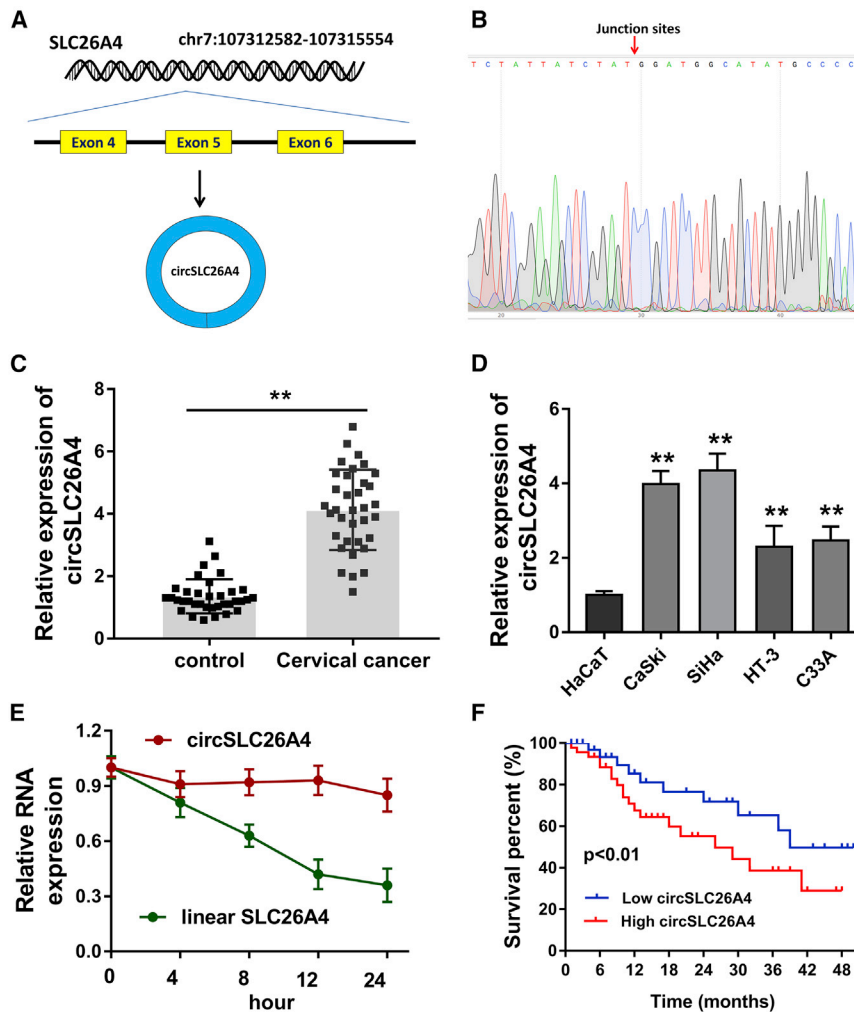**E-mail:** zhuyfdoctor@sina.com
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Figure 1. circRNA circSLC26A4 Is Overexpressed in Cervical Cancer Tissue and Cells

(A) Schematic diagram showed that circSLC26A4 was originated from exons 4, 5, and 6 of the SLC26A4 gene. The genomic location of circSLC26A4 is chr7:107312582-107315554, and the circBase ID is hsa_circ_0132980. (B) Sanger sequencing revealed the back-splicing junction site of exon 6 toward exon 4 in the RNA extraction. (C) RT-PCR indicated the circSLC26A4 expression in cervical cancer tissue and adjacent tissue using divergent primers. (D) RT-PCR indicated the circSLC26A4 expression in cervical cancer cell lines (CaSki, SiHa, HT-3, C33A) and the human epidermal cell (HaCaT). (E) Transcript half-life of circSLC26A4 and its linear transcript in SiHa cells treated with the transcription inhibitor actinomycin D. (F) Survival rate analysis for cervical cancer was performed using the Kaplan-Meier method and log-rank test. $**p < 0.01$.

The divergent primers for circSLC26A4 were synthesized for the qRT-PCR. Data suggested that circSLC26A4 was upregulated in the collected cervical cancer specimens compared to the adjacent normal tissue (Figure 1C). Moreover, the circSLC26A4 expression was also upregulated in cervical cancer cell lines (CaSki, SiHa, HT-3, C33A) compared to human epidermal cell (HaCaT) (Figure 1D). RT-PCR indicated that the transcript half-life of circSLC26A4 was longer than the linear transcript (Figure 1E). Survival analysis for cervical cancer was performed using the Kaplan-Meier method and log-rank test, indicating the poor prognosis of cervical cancer patients with high circSLC26A4 expression (Figure 1F; Table 1). Thus, the data suggest that circRNA circSLC26A4 is overexpressed in cervical cancer tissue and cells, which might act as a risk factor for cervical cancer.

circSLC26A4 Silencing Inhibits the Proliferation, Invasion, and Tumor Growth of Cervical Cancer Cells

The short hairpin RNA (shRNA) targeting circSLC26A4 (sh-circSLC26A4) was synthesized for the loss-of-function experiments

(Figure 2A). The proliferation of cervical cancer cells (CaSki, SiHa) was performed using the colony formation assay, suggesting that circSLC26A4 knockdown inhibited the proliferation (Figures 2B and 2C). The invasion of cervical cancer cells was performed using the Transwell assay, indicating that circSLC26A4 knockdown inhibited the invasion (Figures 2D and 2E). Then, the proliferation of cervical cancer cells was detected using the cell counting kit 8 (CCK-8) assay, showing that circSLC26A4 knockdown inhibited proliferation (Figure 2F). The *in vivo* mice heterotransplantation assay suggests that circSLC26A4 silencing could effectively repress the tumor growth (Figure 2G). In conclusion, circSLC26A4 silencing inhibits the proliferation, invasion, and tumor growth of cervical cancer cells.

circSLC26A4 Sponges the miR-1287-5p in Cervical Cancer Cells

Regarding the molecular mechanism, we found that circSLC26A4 might act as a miRNA sponge to mediate the cervical cancer tumor phenotype. Circular RNA Interactome (CircInteractome) (https://circinteractome.nia.nih.gov/) suggested that miR-1287-5p shared the complementary binding sites with circSLC26A4, and the interaction within the circSLC26A4 and miR-1287-5p was functionally verified by the luciferase reporter assay (Figure 3A). In cervical cancer cell lines (CaSki, SiHa), the miR-1287-5p level was increased with circSLC26A4 knockdown (Figure 3B). RNA-fluorescence *in situ* hybridization (RNA-FISH) showed that circSLC26A4 and miR-1287-5p were both colocated in the cytoplasm of the cervical cancer cell (Figure 3C). The Gene Expression Profiling Interactive Analysis (GEPIA) dataset (http://gepia.cancer-pku.cn/index.html), based on the The Cancer Genome Atlas (TCGA) (http://gepia.cancer-pku.cn), provided strong data that the high miR-1287-5p expression was

Table 1. Relationship between circSLC26A4 and Clinicopathological Characteristics of Cervical Cancer Patients

		circSLC26A4 Expression		
		Low = 16	High = 19	p
Age				
<40 years	19	9	10	0.598
≥40 years	16	7	9	
FIGO Stages				
I–II	13	4	9	0.087
III–IV	22	12	10	
Distant Metastasis				
Yes	17	8	9	0.741
No	18	8	10	
Tumor Size				
<4 cm	9	5	4	0.001*
≥4 cm	26	11	15	
Lymphatic Metastasis				
Yes	22	14	8	0.127
No	13	2	11	

FIGO, International Federation of Gynecology and Obstetrics.
*p < 0.05.

correlated with the better prognosis of cervical cancer individuals (Figure 3D). To identify the interaction within miR-1287-5p and circSLC26A4, RT-PCR data found that miR-1287-5p was negatively correlated with circSLC26A4 in cervical cancer individuals (Figure 3E). Therefore, circSLC26A4 sponges the miR-1287-5p in cervical cancer cells.

HOXA7 Acts as the Target of circSLC26A4/miR-1287-5p

The expression of HOXA7 in cervical cancer tissue specimens was found to be upregulated based on the GEPIA dataset (http://gepia.cancer-pku.cn/index.html) (Figure 4A). Bioinformatics illustrated that miR-1287-5p might share the complementary binding sites with the 3′ UTR of HOXA7 mRNA. Luciferase reporter assay proved that miR-1287-5p could indeed target the HOXA7 with the complementary binding (Figure 4B). RT-PCR illustrated that the miR-1287-5p mimics transfection could decrease the HOXA7 mRNA expression, whereas the miR-1287-5p inhibitor could upregulate the HOXA7 mRNA. Moreover, the cotransfection of the miR-1287-5p inhibitor and circSLC26A4 shRNA could rescue the expression (Figure 4C). Western blot analysis presented that the miR-1287-5p inhibitor could upregulate the HOXA7 protein, and the circSLC26A4 shRNA cotransfection could recover the level (Figures 4D and 4E). Therefore, the data suggest that HOXA7 acts as the target of circSLC26A4/miR-1287-5p.

RBP QKI Promotes the Biogenesis of circSLC26A4

Previous literature has indicated that the RBP QKI could activate the biogenesis and circularization of circRNA in tumorigenesis.[13,14]

Therefore, we tried to identify whether QKI could promote the biogenesis of circSLC26A4 in cervical cancer. Genomic analysis found that there are several QKI response elements (QREs) in intron 3 and intron 6 that are the flanking intron of circSLC26A4 (Figure 5A). RBP immunoprecipitation (RIP) assay showed that QKI could efficiently contact the two QREs in the flanking intron (Figure 5B). Then, we constructed several vectors fused with a mutant of QREs (Figure 5C) and detected the expression of circSLC26A4 using qRT-PCR. Results showed that the expression of circSLC26A4 in the 4# vector, which contains both QREs in the flanking introns, was significantly upregulated compared to others (Figure 5D). Moreover, qRT-PCR showed that the overexpression of QKI could upregulate the circular form of circSLC26A4 rather than the linear form, suggesting that QKI could indeed activate the biogenesis of circSLC26A4 by binding with the specific QREs in the flanking intron (Figure 5E). Clinically, the expression of circSLC26A4 was positively correlated with that of QKI (Figure 5F). Overall, these data support that QKI promotes the biogenesis of circSLC26A4, and circSLC26A4 targets miR-1287-5p/HOXA7 to promote cervical cancer tumorigenesis (Figure 6).

DISCUSSION

Increasing evidence illustrates that circRNAs play a crucial oncogenic or anti-cancer role in the human tumor, such as glioma,[15,16] non-small cell lung carcinoma (NSCLC),[17] breast cancer,[18,19] gastric cancer,[20] et al. Unlike long noncoding RNA and miRNA, circRNAs are characterized by the covalently closed loops without a 3′ end or 5′ end. Moreover, the stable structure of circRNA resisting for the environmental degradation.[21–25]

In this research, we identified a novel circRNA, circSLC26A4, in cervical cancer cells. Results showed that circSLC26A4 was significantly upregulated in cervical cancer tissues and cells. In cellular experiments, circSLC26A4 knockdown inhibited the proliferation, invasion, and tumor growth. In addition, the circSLC26A4 abnormal high expression could be closely correlated with the bad clinical outcome of cervical cancer patients. Overall, these results prove that the circSLC26A4 might function as a vital oncogene for cervical cancer and the valuable therapeutic target for treatment. Besides, the unique structural features of circRNA make it survive in the complicated microenvironment.

Up until now, more and more researchers focus on the expression profile and biological functions of circRNAs in human diseases, especially cancers. In cervical cancer, numerous circRNAs have been identified to participate in the oncogenesis.[26,27] For instance, circRNA circ_0023404 acts as the sponge of miR-136, and then miR-136 targets the TFCP2 to activate the YAP signaling pathway to enforce development and progression.[28] For another example, hsa_circ_0000263 regulates the expression of MDM4 by affecting miR-150-5p, which regulates the p53 gene. hsa_circ_0000263 knockdown inhibits the cell proliferation and migration ability.[29] hsa_circRNA_101996 is highly expressed in cervical cancer tissues compared to corresponding normal tissues, which are positively correlated with TNM stage, tumor size, and lymph node metastasis.
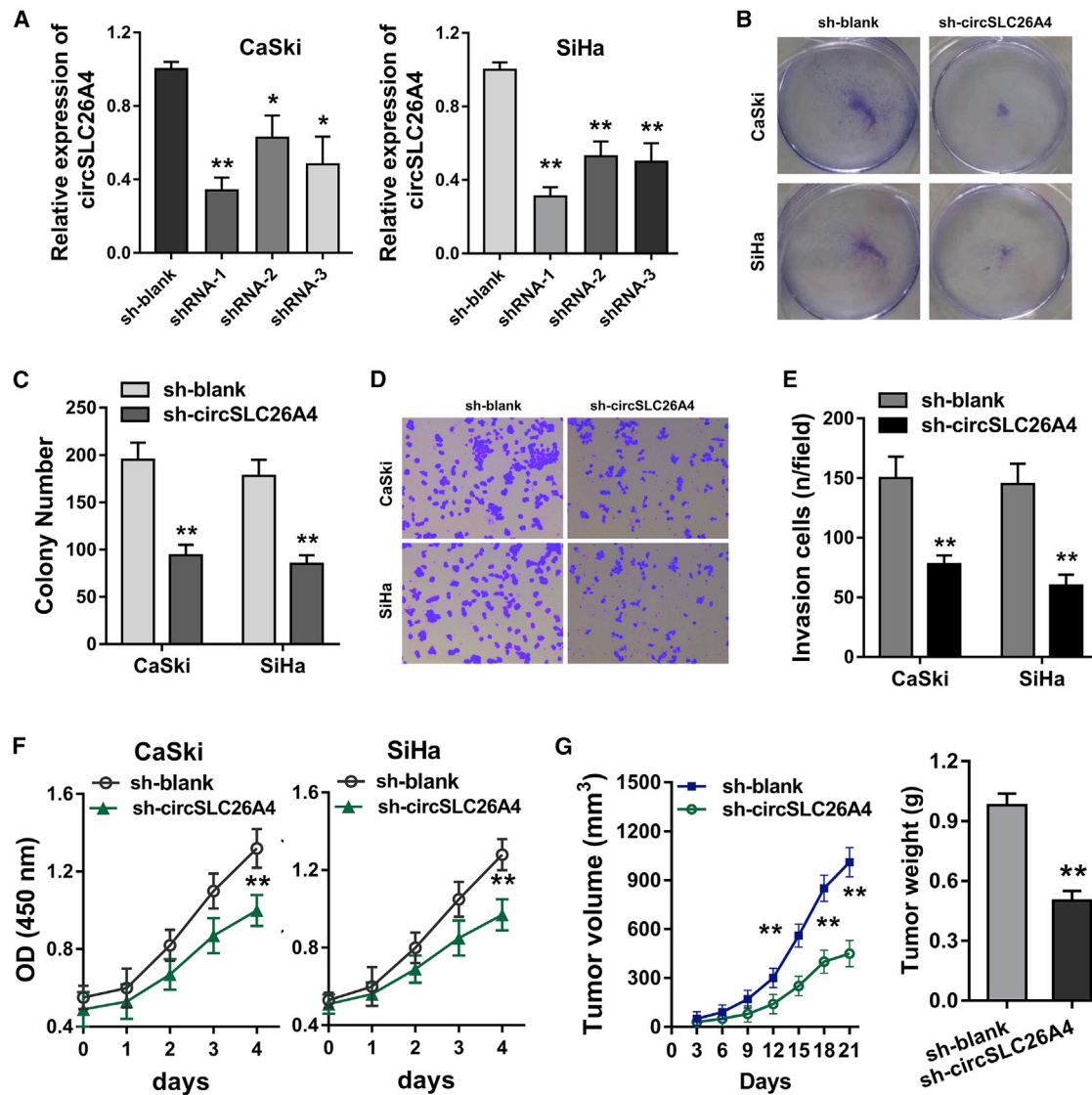
Figure 2. circSLC26A4 Silencing Inhibited the Proliferation, Invasion, and Tumor Growth of Cervical Cancer Cells

(A) The shRNA targeting circSLC26A4 was synthesized for the loss-of-function experiments. (B) The colony formation assay was performed for proliferation of cervical cancer cell lines (CaSki, SiHa). (C) Cell count was presented. (D) The Transwell assay was performed for invasion of cervical cancer cells. (E) Invasion count was presented. (F) The CCK-8 assay was performed for proliferation of cervical cancer cells. (G) *In vivo* mice heterotransplantation assay suggested the tumor growth with circSLC26A4 silencing. **p < 0.01.
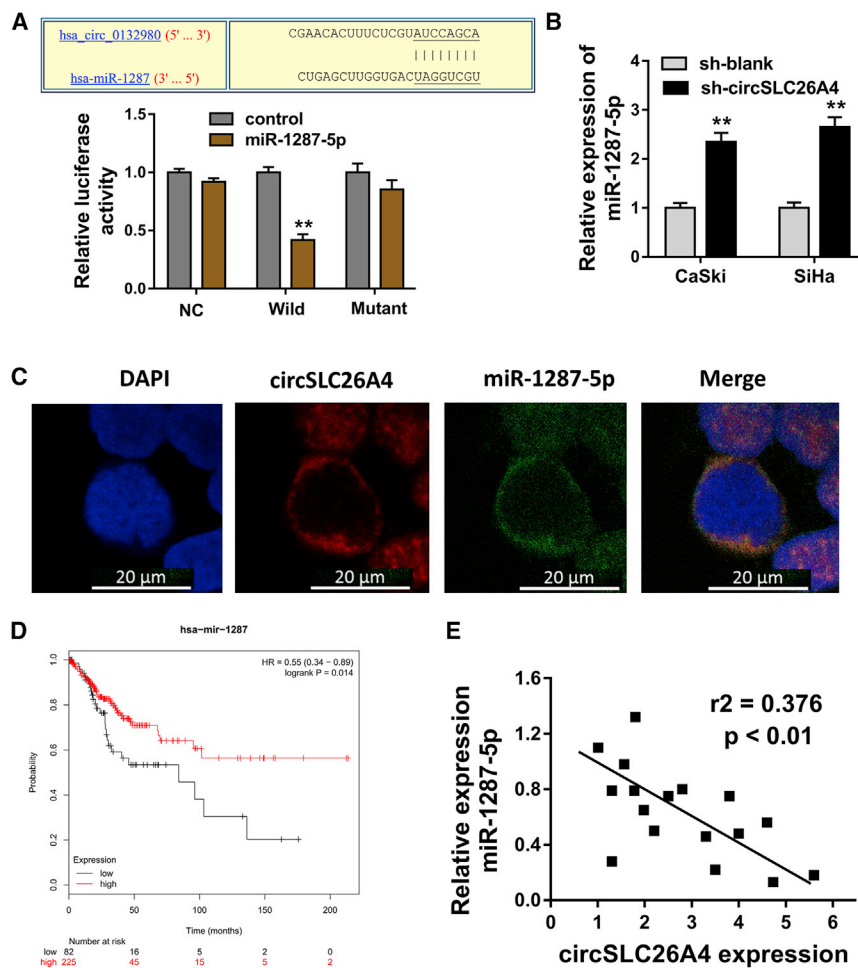
The regulatory mechanism for circRNAs could be concluded as competing endogenous RNA (ceRNA) or others. The mechanism of ceRNA summarizes that circRNAs absorb the miRNAs and then indirectly regulate the target protein of miRNA, forming the circRNA/miRNA/protein regulatory pathway. For example, circRNA circ_0005576 serves as a sponge of miR-153-3p to increase kinesin family member 20A (KIF20A) expression.[30] Therefore, the ceRNA, mediated by circRNA, could efficiently regulate the cervical cancer progress.

HOXA7 is a member of the HOX protein family. It has been reported that HOXA7 is upregulated in the liver cancer tissue, and a higher expression level of HOXA7 is associated with poorer prognosis. In cervical cancer, we also found that HOXA7 is upregulated in the cells. In the mechanism, HOXA7 acts as the target of circSLC26A4/miR-1287-5p.

Regarding the biogenesis of circRNA, we found that RBP QKI could interact with the QREs of a flanking intron of intron 3 and intron 6 in the SLC26A4 gene. This finding helps us to understand the formation mechanism for the circRNA mediated by RBP in cancer.[31–33] In Figure 6, the schematic diagram showed that QKI promotes the biogenesis of circSLC26A4, and circSLC26A4 targets miR-1287-5p/HOXA7 to promote cervical cancer tumorigenesis, forming the QKI/circSLC26A4/miR-1287-5p/HOXA7 axis.

A

hsa_circ_0132980 (5' ... 3')   CGAACACUUUCUCGUAUCCAGCA
                                 | | | | | | | |
hsa-miR-1287 (3' ... 5')   CUGAGCUUGGUGACUAGGUCGU

B

C

DAPI | circSLC26A4 | miR-1287-5p | Merge

D

hsa-mir-1287

E

Figure 3. circSLC26A4 Sponges the miR-1287-5p in Cervical Cancer Cells

(A) CircInteractome (https://circinteractome.nia.nih.gov/) suggested the complementary binding sites with circSLC26A4 and miR-1287-5p. Luciferase reporter assay functionally verified the interaction within the circSLC26A4 and miR-1287-5p. (B) RT-PCR detected the miR-1287-5p level in cervical cancer cell lines (CaSki, SiHa) with circSLC26A4 knockdown. (C) RNA-fluorescence *in situ* hybridization (RNA-FISH) showed the colocation of circSLC26A4 and miR-1287-5p in the cytoplasm of cervical cancer cell. (D) The prognosis of cervical cancer individuals provided the GEPIA dataset based on the TCGA (http://gepia.cancer-pku.cn). (E) The negative interaction within miR-1287-5p and circSLC26A4. **p < 0.01.

Academy of Sciences Cell Bank (Shanghai, China) and culture in RPMI-1640 medium (Invitrogen). Medium was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Hyclone) at 37°C in a humidified atmosphere with 5% CO_2. The oligonucleotides for the circRNA and miRNA were provided by RiboBio (Guangzhou, China). The transfection was performed using Lipofectamine 2000 (Invitrogen). The sequences were presented in Table S1.

qRT-PCR

After the extraction of total RNA from the cells and tissue using TRIzol reagent, the cDNA was synthesized using random oligo or stem-loop primers by the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) for RNAs (circRNA, miRNA, mRNA). RT-PCR was carried out with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI7500 Real-Time PCR instrument (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) acted as the control. The primers for circRNA, miRNA, and mRNA were presented in Table S1.

Sanger Sequencing

After RNA extraction using the TRIzol reagent (Invitrogen), according to the manufacturer's protocol from cervical cancer cells and reversely transcribed, Sanger sequencing was performed by GenePharma (Shanghai, China).

CCK-8 Analysis

The proliferation rate of cervical cancer cells was measured by the CCK-8 assay (Dojindo, Kumamoto, Japan). 5×10^3 Cells/well were seeded in a 96-well flat-bottomed plate for 24 h, and 10 μL of CCK-8 with 100 μL serum-free medium was added to each well. After incubation, the absorbance at 450 nm was measured using an automatic microplate reader (BioTek, Winooski, VT, USA).

In conclusion, our finding confirms that circSLC26A4 functions as an oncogene in cervical cancer tumorigenesis, acting as a miRNA sponge, forming the QKI /circSLC26A4/miR-1287-5p/HOXA7 axis. These results might provide a potential biomarker and insight for the ceRNA mechanism in cervical cancer.
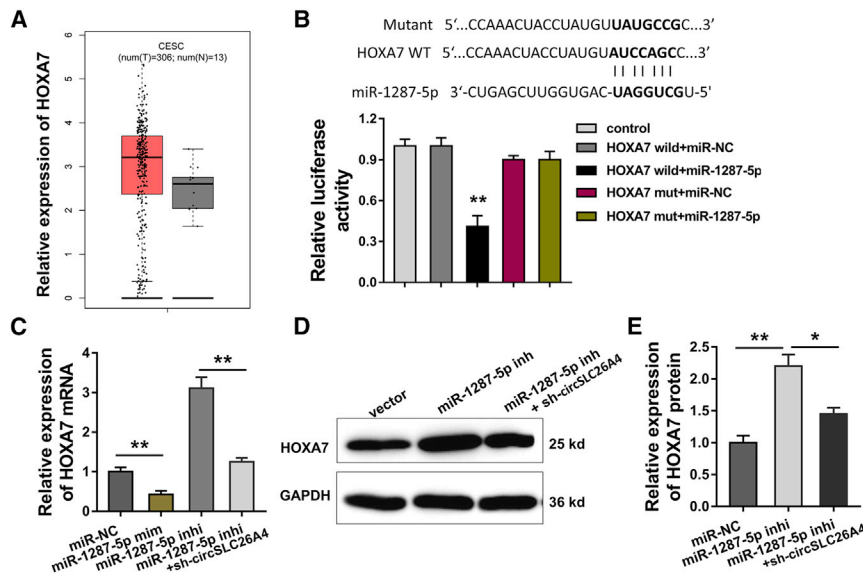
MATERIALS AND METHODS

Patients Recruitment and Samples Collection

The cervical cancer patients (thirty-five cases) were recruited from Jinan University Affiliated Baoan District Maternal and Child Health Care Hospital. All of the volunteers had been informed of the experimental procedure and purpose of this clinical recruitment. Informed consent was acquired from every volunteer. During the surgery, cervical cancer tissue and paired nontumorous tissues were collected. The procedure was approved by the Biomedical Ethics Committee of Jinan University Affiliated Baoan District Maternal and Child Health Care Hospital.

Cell Culture and Transfection

Cervical cancer cell lines (CaSki, SiHa, HT-3, C33A) and human epidermal cell (HaCaT) were purchased from the Chinese

the invaded cell number was counted under microscope (Olympus, Tokyo, Japan).

RNA-FISH

To detect the subcellular location of circRNA and miRNA, RNA-FISH was performed using the fluorescent *in situ* hybridization kit, according to the manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA, USA). The nucleus was counterstained with DAPI. Cy3-labeled circSLC26A4 probes and FAM-labeled miR-1287-5p probes were incubated. The images were obtained with a confocal microscope (Olympus, Japan).

Dual-Luciferase Reporter Assay

For the dual-luciferase reporter assay, the sequence of circSLC26A4, corresponding to miR-1287-5p, including wild type and mutant, were generated and fused into the luciferase vector psi-CHECK-2 (Promega, Madison, WI, USA). Then, the vectors were transfected into 293T cells that seeded in 96-well plates (1×10^4 cells per well). Luciferase reporter vectors were cotransfected with miRNA mimics using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). After 48 h of incubation, firefly activity was measured compared to the Renilla control using a dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions.

RIP Assay

To identify further the interaction within QKI and QREs, RIP was performed using the EZMagna RIP RNA-binding protein immunoprecipitation kit (Millipore, USA), according to the manufacturer's protocols. In brief, cells were lysed using RNA lysis buffer containing RNase and protease inhibitor and then incubated with the RIP buffer containing anti-QKI-coated magnetic beads (Millipore) or immunoglobulin G (IgG; negative control) (Millipore, USA) at 4°C for 2 h. QRE fractions in the precipitates were assayed by qRT-PCR.

In Vivo Mice Assay

The shRNA targeting the circSLC26A4 was packed by the lentiviral vector for stable transfection. Ten BALB/c nude mice (male, 5 weeks) were subcutaneously injected with the transfected cervical cancer cells

Western Blot

Protein extraction from cervical cancer cells was performed by a protein extraction kit (Key Gene, China), following the manufacturer's protocol. Protein concentration was tested by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Protein samples separated by electrophoresis and SDS-containing polyacrylamide gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked and incubated with the primary antibody (anti-HOXA7; Abcam) at 4°C overnight. Afterward, membrane was washed with Tris-buffered saline-Tween 20 (TBST) buffer, three times over 10 min, and incubated with the horseradish peroxidase (HRP)-labeled secondary antibody at room temperature for 2 h. Lastly, blot signal was visualized by an enhanced chemiluminescence (ECL) detection system (Bio-Rad, Hercules, CA, USA).

Colony Proliferation Assay

The transfected cervical cancer cells (CaSki, SiHa) with shRNA or controls were seeded in six-well plates at the density of 1×10^3 cells/well. After 14 days, the cells were fixed with 4% paraformaldehyde (30 min) and stained with 0.1% crystal violet (30 min) (Solarbio, Shanghai). Colonies more than 500 cells were counted.

Transwell Assay

The invasion ability of cervical cancer cells (CaSki, SiHa) was detected using the Transwell inserts (8 μm pore size; Costar, Cambridge, MA), precoated with Matrigel (BD Biosciences, San Jose, CA). About 1×10^5 cervical cancer cells were cultured in serum-free medium and then seeded into the upper chamber. The complete DMEM was filled in the bottom chamber. After incubation, the cells that passed through the filter were fixed and stained with 4% paraformaldehyde and stained with 0.1% crystal violet. Five visual fields were randomly selected, and
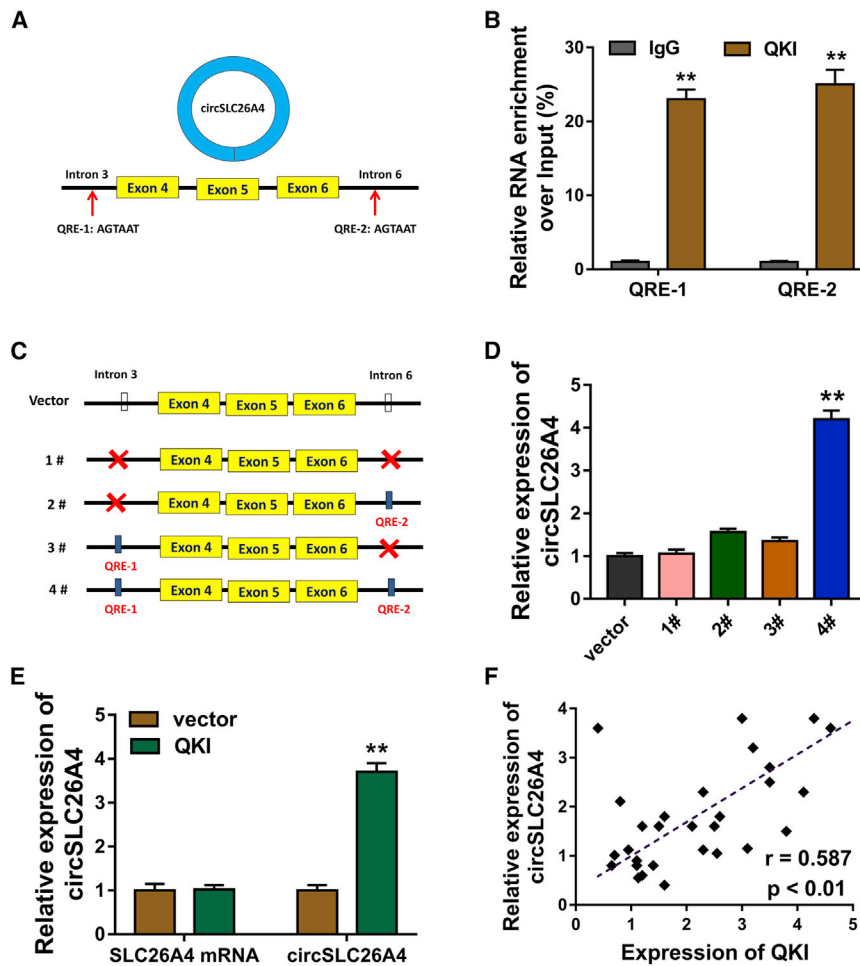
Figure 5. RNA Binding Protein Quaking (QKI) Promotes the Biogenesis of circSLC26A4

(A) Schematic diagram indicated the QKI response elements (QREs) in the intron 3 and intron 6. (B) RNA binding protein immunoprecipitation (RIP) assay showed the binding of QKI on the two QREs in the flanking intron. (C) Schematic diagram indicated the vectors fused with mutant of QREs. (D) qRT-PCR showed the expression of circSLC26A4 in vectors. (E) qRT-PCR showed the expression of the circSLC26A4 linear form and circular form. (F) The correlation within expression of circSLC26A4 and QKI. **$p < 0.01$.
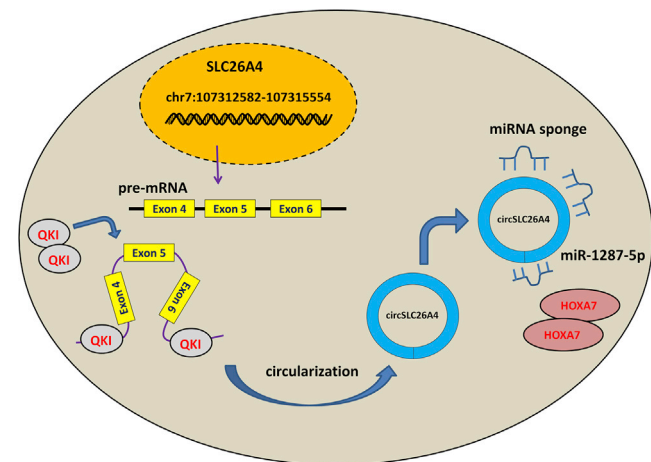
Figure 6. Schematic Diagram Showed that QKI Promotes the Biogenesis of circSLC26A4, and circSLC26A4 Targets miR-1287-5p/HOXA7 to Promote Cervical Cancer Tumorigenesis, Forming the QKI/circSLC26A4/miR-1287-5p/HOXA7 Axis

(SiHa, 10^6 cells, 100 μL). After the injection, the length and width of the neoplasm were measured every 3 days. After 3 weeks, the mice were sacrificed, and the neoplasm was weighed. The animal experiments were performed in accordance with the guidelines for the Care and Use of Laboratory Animals and approved by Jinan University Affiliated Baoan District Maternal and Child Health Care Hospital.

Statistical Analyses

The statistical analysis was presented as the mean ± SEM and calculated by SPSS version 19.0 (IBM SPSS, Chicago, IL, USA) and Prism GraphPad version 6.0 (GraphPad, LaJolla, CA, USA). The statistical significance was calculated using an unpaired Student's t test. Survival rate analysis was performed using the Kaplan-Meier method and log-rank test. p value < 0.05 was regarded as statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.11.032.

AUTHOR CONTRIBUTIONS

Fei Ji, Rong Du performed this assays. Tianfeng Chen, Meng Zhang act as assists. Yuanfang Zhu, Xin Luo, Yan Ding performed as leader.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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