Circular RNA HIPK3 plays a carcinogenic role in cervical cancer progression via regulating miR-485-3p/FGF2 axis

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molecule with a stable closed-loop structure. The

circular RNA HIPK3 (circHIPK3) is highly expressed

in hepatocellular carcinoma and facilitates tumor

growth. However, its role in cervical cancer (CC)

This study aimed for investigating the function of

circHIPK3 on proliferation and metastasis of CC

in CC cell lines. Cell counting kit-8 and colony

of overexpression and knockdown of circHIPK3

on CC cell proliferation. Dual-luciferase reporter

assay was employed to probe into the binding of

miR-485-3p to circHIPK3 and miR-485-3p to the 3'

and its regulatory mechanisms are not well-studied.

cells. In this study, quantitative real-time PCR assay

was adopted to delve into the circHIPK3 expression

formation assays were used to evaluate the influence

ABSTRACT Circular RNA (circRNA) is an endogenous RNA

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To cite: Wu S, Liu S, Song H, et al. J Investig Med Epub ahead of print: [please include Day Month Year]. doi:10.1136/jim-2020-001537 untranslated region (UTR) of fibroblast growth factor 2 (FGF2), respectively. FGF2 protein expression was detected by western blot analysis. This study confirmed that circHIPK3 was highly expressed in CC tissues. Overexpressed circHIPK3 could remarkably expedite the proliferation, migration and invasion of SiHa cells, and knocking down circHIPK3 could significantly impede the proliferation, migration and invasion of HeLa cells. MiR-485-3p can directly bind to circHIPK3 and the 3'UTR of FGF2. Overexpression of circHIPK3 triggered the upregulation of FGF2 expression while knockdown of circHIPK3 reduced FGF2 expression in CC cells, and the transfection of miR-485-3p mimics reversed the upregulation of FGF2 expression and enhanced malignant phenotypes in CC cells with overexpressed circHIPK3. INTRODUCTION Cervical cancer (CC) is one of the most common gynecological tumors.¹ At present, although a variety of methods are employed to treat CC, including surgery, radiotherapy and chemotherapy, distant metastasis and recurrence still result in poor prognosis and low survival rate of the patients.² Therefore, studying its molecular mechanism and exploring new therapeutic

targets are essential for the treatment of CC. Circular RNA (circRNA), a kind of noncoding RNA, is widely present in mammals.^{3–5} Most of the circRNAs are derived from the exon regions of genes and few are formed by intron splicing.^{6–7} Unlike long non-coding RNAs and

Significance of this study

What is already known about this subject?

- Cervical cancer (CC) is one of the leading cancer-related death causes all over the world.
- Circular RNAs (circRNAs) participate in gene regulation and tumor progression.
- Circular RNA HIPK3 (circHIPK3) is highly expressed in hepatocellular carcinoma and facilitates tumor growth.

What are the new findings?

- CircHIPK3 expression was remarkably upregulated in CC tissues.
- Overexpression circHIPK3 could significantly promote the proliferation, migration and invasion of SiHa cells, and knockdown circHIPK3 could remarkably inhibit the proliferation, migration and invasion of HeLa cells.
- CircHIPK3 played a carcinogenic role in CC progression via regulating miR-485-3p/ FGF2 axis.

How might these results change the focus of research or clinical practice?

These results help understand the mechanism underlying CC pathogenesis, and may promote the development of therapeutical approaches for treating CC.

microRNAs (miRNAs), circRNAs do not have a 5' end and a 3' end but possess a covalently closed cyclic structure.⁸⁹ circRNA is involved in the physiological and pathological regulation of human cells. Circular RNA HIPK3 (circHIPK3, hsa_circ_0000284) is highly expressed in liver, brain and lung, and it mainly originates from the second exon of gene HIPK3. In liver cancer, circHIPK3 functions as a 'sponge' to adsorb miR-124 and upregulates target gene expressions of IL6R and DXL2 through competitive endogenous RNA (ceRNA) mechanism, thus promoting cancer progression.¹⁰ However, the expression, function and mechanism of circHIPK3 in CC have not been clarified.

miRNAs are non-coding single-stranded RNAs consisting of 20–25 nucleotides. miRNAs can bind to the 3' untranslated region (UTR) of

Original research

mRNA, degrade mRNA or impede its translation process, thereby modulating the expressions of the target genes.^{11 12} Besides, miRNA exerts a crucial regulatory function in the progression of cancer.¹³ Previous studies authenticate that the abnormal expression of miR-485-3 p is closely related to tumorigenesis and miR-485-3 p functions as a tumor suppressor in osteosarcoma, breast cancer, prostate cancer, glioblastoma and other malignant tumors,^{14–17} but the role of miR-485-3 p in CC still awaits more investigations.

In this work, we studied the function of circHIPK3 in CC and found that circHIPK3 could promote the cell proliferation and metastasis of CC cells through regulating miR-485-3 p/fibroblast growth factor 2 (FGF2) axis.

MATERIALS AND METHODS Tissue samples

The tumor tissues and matched non-tumor tissues of 70 patients with CC in Linyi Cancer Hospital from December 2016 to August 2019 were obtained. All patients with CC did not receive chemotherapy or radiation before surgery. After the tissues were removed during surgery, all samples were immediately stored at -80° C for further analysis.

Cell culture

The China Center for Type Culture Collection (Wuhan, China) was the provider of human CC cell lines (SiHa and HeLa). CC cells were cultured in RPMI-1640 medium (HyClone, Logan, Utah, USA) containing 10% inactivated fetal bovine serum (FBS; HyClone), 100 U/mL penicillin and 0.1 mg/mL streptomycin (HyClone) at 37°C in 5% CO₂ and saturated humidity, and passaged at the interval of every 2–3 days. The cells in the logarithmic growth phase were harvested for the following experiments.

Quantitative real-time PCR assay

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Shanghai, China). RNA was reversely transcribed into complementary DNA using PrimeScript RT Reagent kit (Invitrogen) in accordance with manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) assay was performed using the Bio-Rad CFX96 qPCR system and

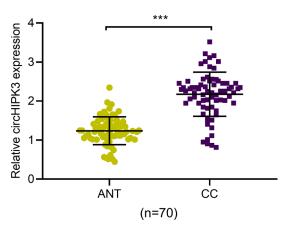


Figure 1 Circular RNA HIPK3 (circHIPK3) expression was upregulated in cervical cancer (CC). The expression of circHIPK3 in 70 pairs of CC tissue and adjacent normal tissues was detected by quantitative real-time PCR (qRT-PCR) assay. ***P<0.001.

SYBR Green Premix Ex Taq II (TaKaRa, Dalian, China) in compliance with the manufacturer's protocol. GAPDH and U6 were used as the internal reference of the expressions of circHIPK3 and miR-485-3 p, respectively, and the quantification was performed by the $2^{-\Delta\Delta Ct}$ method. The sequences of specific primers were as follows:

circHIPK3-F: 5'-CTACAGATCCGACCAGGAGTTC-3'; circHIPK3-R: 5'-TGTGAACCAGCCACACTCTCAG-3'; miR-485-3 p-F: 5'-GCCGAGGUCAUACACGGCUCU-3'; miR-485-3 p-R: 5'-CTCAACTGGTGTCGTGGA-3'; GAPDH-F: 5'-GGAATCCACTGGCGTCTTCA-3'; GAPDH-R: 5'-GGTTCACGCCCATCACAAAC-3'; U6-F: 5'-GCTTCGGCAGCACATATACTAA-3'; U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Cell transfection

The circRNA overexpression vector pLCDH-circRNA was purchased from Guangzhou Geneseed Biotech, and the circHIPK3 sequence was obtained from the circBank database. Then circHIPK3 sequence fragment was inserted into pLCDH-circRNA. Small interfering RNA (siRNA) targeting circHIPK3, miR-485-3 p mimics and miR-485-3 p inhibitors were constructed by GenePharma (Shanghai, China). Cells were transfected with pLCDH-circHIPK3, siRNAs, miRNA mimics and inhibitors with Lipofectamine 3000 (Invitrogen; ThermoFisher Scientific) according to the supplier's instructions. Finally, the transfection efficiency was detected by qRT-PCR assay.

Cell proliferation assay

Cells in the logarithmic growth phase were trypsinized with 0.25% trypsin to prepare single-cell suspension. Cells were counted, inoculated in a 96-well plate at the density of 2000 cells/well and cultured for 12, 24, 48, 72 and 96 hours, respectively. On each day, after 10 μ L/well cell counting kit-8 (Dojindo, Kumamoto, Japan) was added, the cells were incubated for 2 hours. After that, the absorbance of each well was detected at the wavelength of 450 nm. Nine-ty-six hours later, the proliferation curve was plotted.

BrdU staining

BrdU assay was also used to detect CC cell proliferation. Cells in each group were inoculated into 24-well plates (with a cover glass) and cultured at a density of 2.5×10^5 cells/well for 24 hours. Then BrdU kit (RiboBio, Guangzhou, China; final concentration: $50 \,\mu$ mol/L) was loaded and incubated at 37° C for 2 hours. After the medium was discarded and the slide was gently washed three times with phosphate-buffered saline (PBS), and the cells were fixed with 4% paraformaldehyde for 30 min. Next, anti-BrdU antibody (Beyotime, Shanghai, China) was added, with which the cells were incubated in the dark for 30 min at room temperature. After that, DAPI staining solution was added and incubated for 30 min, and then the cells were gently washed three times with PBS. Eventually, the images were obtained under fluorescence microscope (Olympus, Tokyo, Japan).

Cell migration and invasion assay

The migration and invasion of CC cells were determined by Transwell assay. To be specific, in the migration assay, 2×10^4 cells resuspended in 200 µL serum-free medium were added

into upper compartment of the Transwell Chambers (Corning, Beijing, China) and $600 \,\mu$ L medium containing 20% FBS was added to the lower upper compartment. After being cultured for 24 hours, the cells in the upper compartment were removed, and cells passing through the membrane were fixed in 4% paraformaldehyde, followed by being stained with 0.1% crystal violet, washed by tapped water, photographed and counted. In the invasion assay, Transwell membrane needed to be coated with Matrigel before the experiments, and in the migration assay, Matrigel was not used.

Dual-luciferase activity assay

Dual-luciferase activity assay was used to verify the target relationship between circHIPK3 and miR-485-3 p, and miR-485-3 p and the 3'UTR of FGF2. HEK-293T cells were inoculated to 24-well plates (5×10^5 cells/well). Wild-type (WT) or mutant-type (MUT) circHIPK3 sequence or FGF2 3'UTR sequence was subcloned to pGL3 basic vector (Promega, Madison, Wisconsin, USA) and then the reporter vectors were transfected into cells. MiR-320 mimics or negative controls were co-transfected into the cells, respectively. Forty-eighthours later, luciferase activity was determined

by using the dual-luciferase reporter system (Promega) in line with the manufacturer's instructions.

Western blot analysis

After the cells were rinsed three times with cold PBS, and RIPA lysate was loaded, with which the cells were sonicated, and the protein concentration was quantified by Bradford assay. The same amount of protein from each group was separated by SDS-PAGE and then the protein on the gel was transferred to PVDF membrane (Beyotime). After that, the membrane was blocked with defatted milk at room temperature for 1 hour, and the primary antibody anti-FGF2 (ab92337, Abcam, 1:1000) and anti-GAPDH (ab181602, Abcam, 1:3000) were loaded and incubated at 4°C overnight. Then the secondary antibody Goat Anti-Rabbit IgG H&L (HRP) (ab205718) (1:2000; Abcam, UK) was added and incubated for 1 hour at room temperature. After that, the membrane was washed three times with TBS+Tween (TBST) and each time lasted for 5 min. Then, super-sensitive enhanced chemiluminescence substrate (Hubei Biossci Biotechnology, Wuhan, China) was added on the membrane to develop the bands employing chemiluminescence method.

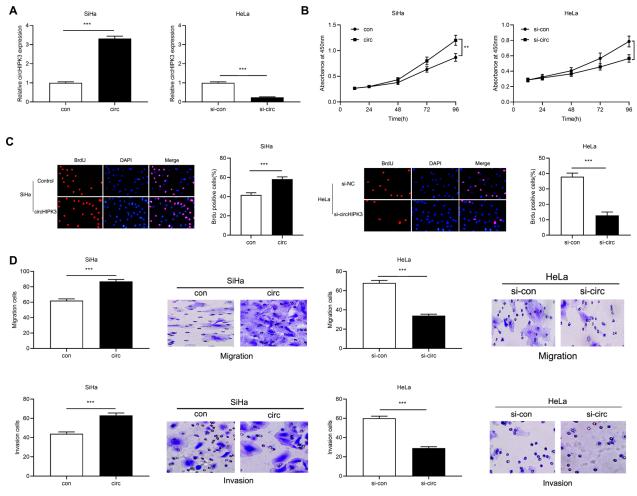


Figure 2 Circular RNA HIPK3 (circHIPK3) facilitated cervical cancer (CC) cell proliferation and metastasis. (A) The expression of circHIPK3 was detected in SiHa and HeLa cells transfected with plasmid or small interfering RNA (siRNA). (B) The proliferation ability of HeLa and SiHa cells after overexpression or knockdown of circHIPK3 was detected by cell counting kit-8 (CCK-8) assay. (C) The cell viability of cells after overexpression or knockdown of circHIPK3 was examined by BrdU staining. (D) The migration and invasion of CC cells were measured by the Transwell assay. *P<0.05, **p<0.01, ***p<0.001.

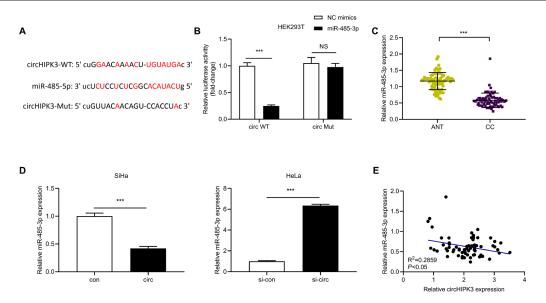


Figure 3 Circular RNA HIPK3 (circHIPK3) targeted miR-485-3 p. (A) The schematic diagram showed the complementary binding sites between circHIPK3 and miR-485-3 p. (B) Luciferase reporter assay showed that miR-485-3 p had a negative regulatory effect on the activity of circHIPK3-WT in HEK293T cells. (C) The expression of miR-485-3 p in cervical cancer (CC) tissue and normal tissue was detected by quantitative real-time PCR (qRT-PCR) assay. (D) The effects of overexpression or knockdown of circHIPK3 on miR-485-3 p expression in SiHa and HeLa cells were determined by qRT-PCR assay. (E) The correlation between circHIPK3 and miR-485-3 p was evaluated by qRT-PCR assay. ***P<0.001. NS: p>0.05.

Statistical analysis

Statistical analysis was conducted using SPSS V.13.0 statistical software (SPSS, Chicago, Illinois, USA). The difference between the two groups was compared by student's t-test. P value < 0.05 was regarded as a significant difference.

RESULTS

circHIPK3 expression was upregulated in CC tissues and circHIPK3 facilitated CC cell proliferation and metastasis

Above all, qRT-PCR assay was performed to detect the expression of circHIPK3 in 70 pairs of CC tissues and adjacent normal tissues. The expression of circHIPK3 in CC tissues was significantly higher compared with in matched non-tumor tissues (figure 1). To explore the role of circHIPK3 in CC, we successfully constructed circHIPK3 overexpression and knockdown cell lines with SiHa and HeLa cell lines, respectively (figure 2A). Then cell counting kit-8 (CCK-8) assay and BrdU assay were used to detect cell proliferation. The results depicted that the proliferation of SiHa cells with overexpressed circHIPK3 was dramatically enhanced compared with the control group; the proliferation of HeLa cells with circHIPK3 knockdown was impeded compared with the control group (figure 2B,C). Transwell assay was performed to determine the migration and invasion of CC cells. Overexpression of circHIPK3 markedly increased the migration and invasion of SiHa cells; in contrast, knockdown of circHIPK3 remarkably decreased migration and invasion of HeLa cells (figure 2D).

circHIPK3 targeted miR-485-3p

StarBase database (www.starbase.sysu.edu.cn) was used to search for miRNA that could potentially bind to circHIPK3. It was found that miR-485-3p was one of the predicted miRNAs, and the binding site was displayed (figure 3A). To further verify whether miR-485-3p can directly bind to circHIPK3, we constructed a luciferase reporter vector containing WT or MUT circHIPK3 sequence. The results manifested that miR-485-3 p significantly reduced the luciferase activity of circHIPK3-WT but did not inhibit the luciferase activity of circHIPK3-MUT (figure 3B). The expression of miR-485-3p in CC was then detected by qRT-PCR assay, the results of which elucidated that the expression of miR-485-3p in CC tissues was markedly lower than that in adjacent tissues (figure 3C). Additionally, we found that upregulation of circHIPK3 expression dramatically reduced miR-485-3 p expression in SiHa cells, whereas knockdown of circHIPK3 worked oppositely in HeLa cells (figure 3D). Importantly, in CC tissues, the expression of circHIPK3 was negatively correlated with the expression of miR-485-3 p (figure 3E). The above data proved that circHIPK3 targeted miR-485-3 p in CC and negatively regulated the expression of miR-485-3 p.

MiR-485-3p reversed the promoting effect of circHIPK3 on the malignant phenotypes of CC cells

Next, we explored whether circHIPK3 exerted its oncogenic function in CC via regulating miR-485-3 p expression. CCK-8 assay uncovered that overexpression of miR-485-3 p dramatically inhibited the proliferation of SiHa cells, while circHIPK3 overexpression could attenuate the inhibitory effect of miR-485-3 p on the proliferation of SiHa cells (figure 4A). Moreover, Transwell assay unmasked that compared with the control group, transfection of miR-142-5 p mimics remarkably reduced migration and invasion of SiHa cells and this inhibitory effect could be counteracted by circHIPK3 overexpression (figure 4B). After miR-485-3 p inhibitors were transfected into HeLa cells, it was found that the proliferation, migration and

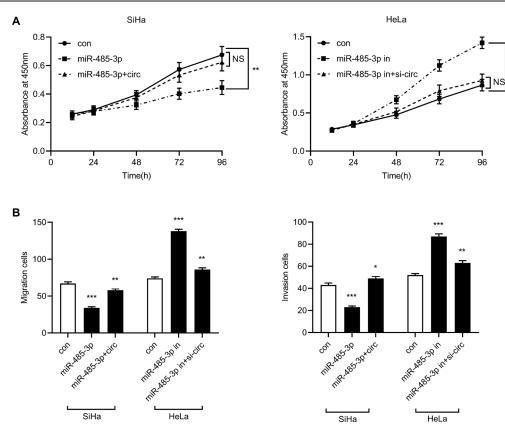


Figure 4 Circular RNA HIPK3 (circHIPK3) reversed the inhibitory effect of miR-485-3 p on cervical cancer (CC) cells. (A) Cell counting kit-8 (CCK-8) assay was used to determine the effect of circHIPK3 overexpression on cell proliferation after the overexpression of miR-485-3 p in SiHa cells and the effect of knocking down circHIPK3 on proliferation after the inhibition of miR-485-3 p in HeLa cells. (B) Transwell assay was used to detect the effect of circHIPK3 overexpression on cell migration and invasion after the overexpression of miR-485-3 p in SiHa cells and the effect of knocking down circHIPK3 on cell migration and invasion after the inhibition of miR-485-3 p in HeLa cells. *P<0.05, **p<0.01, ***p<0.001. NS: p>0.05.

invasion of HeLa cells were increased, and knockdown of circHIPK3 significantly reversed these effects (figure 4A,B). Collectively, circHIPK3 promoted the proliferation, migration and invasion of CC cells via regulating miR-485-3 p.

FGF2 was a target gene of miR-485-3p and was indirectly regulated by circHIPK3

To expound the 'ceRNA' mechanism among circHIPK3, miR-485-3 p and downstream target gene in CC, we used TargetScan and StarBase databases to predict potential target genes of miR-485-3 p and found that miR-485-3 p had complementary sites with 3'UTR of FGF2 (figure 5A). qRT-PCR assay data depicted that the expression of FGF2 in CC tissues was negatively correlated with the expression of miR-485-3 p while positively associated with the expression of circHIPK3 (figure 5B,C). Then dual-luciferase activity assay was performed. As shown, miR-485-3 p inhibited luciferase activity of WT FGF2 3'UTR but no obvious change could be observed on the luciferase activity of mutated sequence (figure 5D). Additionally, after the transfection with miR-485-3 p mimics, there was a decrease in FGF2 at both mRNA and protein levels in SiHa cells and this effect was attenuated by circHIPK3 overexpression; after the transfection with miR-485-3 p inhibitors, there was an increase in FGF2 at both mRNA and protein levels in HeLa cells and this effect was counteracted by circHIPK3

knockdown (figure 5E,F). These data confirmed that FGF2 was a direct target of miR-485-3 p and that FGF2 was negatively regulated by miR-485-3 p but positively modulated by circHIPK3.

DISCUSSION

circRNA participates in tumorigenesis and can affect tumor cell proliferation, apoptosis and metastasis.¹⁸ For example, circ 0044516 can function as an oncogenic factor to promote proliferation and metastasis of prostate cancer cells¹⁹; circRNA-SMARCA5 expression is downregulated in multiple myeloma, and circRNA-SMARCA5 inhibits tumor cell proliferation and promotes apoptosis²⁰; circAPC impedes the proliferation of diffuse B-cell lymphoma cells by inactivating wnt/ β -catenin signaling pathway through the interaction with TET1 and miR-888²¹; in hepatocellular carcinoma, circ-IGF1R promotes the proliferation of hepatocellular carcinoma cells and inhibits apoptosis by activating the PI3K/AKT pathway.²² In this study, we found that circHIPK3 was highly expressed in CC tissues. Overexpression of circHIPK3 significantly promoted the proliferation, migration and invasion of CC cells, while knocking down circHIPK3 had the opposite effect. These findings revealed the importance of circHIPK3 in the progression of CC.

miRNAs can directly bind to the target mRNA and induce mRNA degradation or inhibit translation.²³ In previous

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previous reports.

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circHIPK3 significantly attenuated this promotion. Therefore, we concluded that circHIPK3 partook in the regulation of

We found a binding site between circHIPK3 and miR-485-3 p

through bioinformatics analysis. Moreover, it was confirmed

by luciferase reporter gene assay that circHIPK3 could adsorb

miR-485-3 p. Additionally, knockdown of circHIPK3 could

contribute to an increase in the expression of miR-485-3 p.

Upregulation of miR-485-3 p expression inhibited prolifera-

tion and metastasis of CC cells, and this inhibition could be

attenuated by circHIPK3. Meanwhile, downregulation of

miR-485-3 p expression significantly promoted the ability of

CC cells to proliferate and metastasize, while knocking down

studies, miR-485-3 p is reported to be a tumor suppressor can adsorb miR-182 to upregulate NPTX1 expression, in multiple human cancers. For example, downregulation thus inhibiting the progression of liver cancer.²⁵ Besides, of miR-485-3 p expression promotes proliferation and circPIP5K1A promotes the progression of colon cancer via inhibiting miR-1273a expression.²⁶ Given that circHIPK3 metastasis of glioblastoma cells via targeting RNF135¹⁷; miR-485-3 p negatively regulates transcriptional co-reand miR-485-3 p exert opposite effects in CC, we supposed pressor CtBP1 to suppress the occurrence and developthat there was a similar targeting relationship between them.

1.0 niR-483-39 IT niR-483-3P miR-483-39 niR-483-30¹¹ 0.5 control *sircifc *citc 0.0 hik-A85-3P HIR-A85-39roite 1 Hik-485-3Pir niR-ABD-3PH Haberto 00 1 Figure 5 Fibroblast growth factor 2 (FGF2) was a target gene of miR-485-3 p and was indirectly regulated by circular RNA HIPK3

ment of osteosarcoma¹⁴; in breast cancer, miR-485-3 p

inhibit cancer cell metastasis through inhibiting PGC-1a

expressions.¹⁵ In the present study, we demonstrated that

miR-485-3 p expression was downregulated in CC, and

overexpression of miR-485-3 p inhibited the proliferation

and metastasis of CC cells while inhibition of miR-485-3 p

exerted the opposite effect, which was consistent with

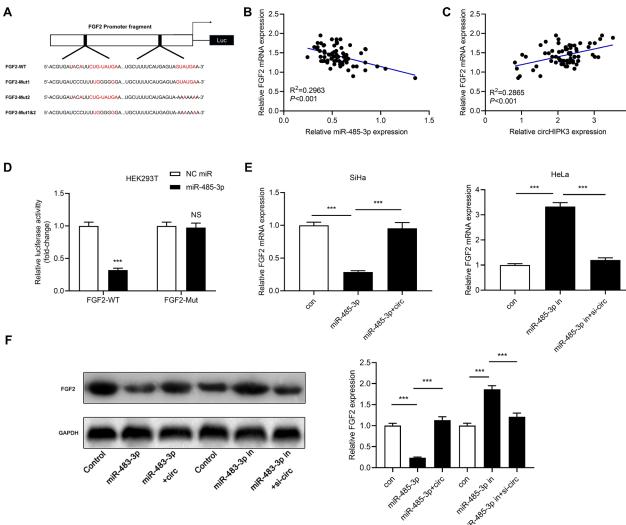
regulating gene expressions.¹⁸ For example, circ 0026344

inhibits the metastasis of colorectal cancer cells by func-

tioning as a miR-183 sponge.²⁴ Circ 0070269, as ceRNA,

It is worth noting that circRNA can act as ceRNA, thereby

(circHIPK3). (A) The schematic diagram showed the complementary binding sites between miR-485-3 p and the 3' untranslated region (UTR) of FGF2 mRNA. (B) Spearman's analysis revealed a negative correlation between miR-485-3 p and FGF2. (C) Spearman's analysis revealed a positive correlation between circHIPK3 and FGF2. (D) Luciferase reporter gene assay was used to detect the binding of miR-485-3 p to FGF2 in HEK293T cells. (E) FGF2 mRNA expression in each group was detected by quantitative real-time PCR (gRT-PCR) assay. (F) FGF2 protein expression in each group was detected by western blot analysis assay. ***P<0.001.



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FGF2 is a member of the fibroblast growth factor family (FGFs) and participates in multiple physiological and pathological processes, promoting embryonic development and cell differentiation, tumor growth, angiogenesis and so on. Reportedly, FGF2 expression is increased in endometrial cancer, and downregulation of FGF2 expression can inhibit tumor cell migration and invasion by inhibiting Wnt/β-catenin signaling pathway.²⁷ In cervical intraepithelial neoplasia tissues, the expression of FGFs is increased, which are positively correlated with clinical stage of CC, and FGF2 overexpression significantly facilitates CC cell proliferation, migration and invasion.²⁸ These data indicate that FGF2 is a crucial modulator during the tumorigenesis and progression of CC. In this study, we observed that inhibition of miR-485-3 p or circHIPK3 overexpression could enhance the expression of FGF2 in CC. Our data partly explained why circHIPK3 was oncogenic and miR-485-3 p was tumor-suppressive in CC.

This work has several limitations. Foremost, this study is only based on in vitro experiments, so in vivo data and clinical data from more medical centers are necessary to further validate our conclusion in the following work; other downstream miRNAs of circHIPK3 in CC needs to be screened and identified; to explore the potential of circHIPK3 as a biomarker, more samples from multiple centers should be included and the survival analysis of patients with CC based on the expression of circHIPK3 is desirable.

In summary, we demonstrate that circHIPK3 is significantly highly expressed in CC tissues. Besides, in vitro studies show that knockdown of circHIPK3 inhibits CC cell proliferation and metastasis, while overexpression of circHIPK3 abrogates these effects. In terms of mechanism, we confirm that circHIPK3 negatively regulates miR-485-3 p but positively regulates FGF2. Our work provides a new theoretical basis for CC progression and clues for the diagnosis and treatment of this disease.

Contributors Conceived and designed the experiments: XZ. Performed the experiments: SW, SL, HS. Statistical analysis: HS. Wrote the paper: SW. All authors read and approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

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