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ORIGINAL ARTICLE

# CircHIPK3 modulates VEGF through MiR-7 to affect ovarian cancer cell proliferation and apoptosis

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## Summary

***Purpose:*** *The purpose of this study was to observe the effects of circHIPK3on the proliferation and apoptosis of ovarian cancer cells, and to further explore the potential mechanism therein.*

***Methods:*** *CircHIPK3 was determined in the carcinoma tissues, normal adjacent tissues, and also in ovarian cancer cells via RT-PCR. The proliferation and apoptosis of cells were observed via colony-forming assay, 5-ethynyl-2'-deoxyuridine (EdU) staining and Western blotting. Moreover, the effect of the inhibition of circHIPK3 on the in vivo growth of ovarian cancer cells was detected using subcutaneous tumorigenesis assay. Finally, the effect of circHIPK3 on the expression of the micro ribonucleic acid (miR)-7/vascular endothelial growth factor (VEGF) signaling pathway in ovarian cancer cells was examined.*

***Results:*** *CircHIPK3 in the carcinoma tissues was obviously higher than that in normal adjacent tissues. SKOV3 cell lines transfected with circHIPK3 inhibitor exhibited declined number of colonies. The inhibition of circHIPK3 distinctly suppressed the expression of B-cell lymphoma 2 (Bcl-2) and raised that of Bcl-2 associated X protein (Bax). Besides, the inhibition of circHIPK3 obviously weakened the tumorigenicity of ovarian cancer cells subcutaneously transplanted. Finally, it was found that miR-7 declined obviously and VEGF rose distinctly in the carcinoma tissues, and the in vitro assay verified the obvious increase in the expression of miR-7 and the prominently inhibited VEGF protein expression in the ovarian cancer cells with the inhibition of circHIPK3.*

***Conclusion:*** *CircHIPK3 has an obviously increased expression level in the carcinoma tissues of ovarian cancer patients, and the inhibition of circHIPK3 can activate the miR-7-mediated decline in the expression of VEGF to repress the proliferation and promote the apoptosis of ovarian cancer cells.*

***Key words:*** *circHIPK3, ovarian cancer, proliferation, apoptosis, miR-7, VEGF*

## Introduction

Ovarian cancer is the fifth most common cause of deaths of gynecological cancer in the United States, and about 14,000 women died of ovarian cancer in 2017 [1]. There were approximately 295,000 cases of ovarian cancer worldwide in 2018, of whom 180,000 patients died. According to the epidemiological investigation, ovarian cancer is affected by stimuli, reproduction, genetics, inflammation and lifestyles [2]. The 5-year survival rate of patients is lower than 35% at the definite diagnosis of ovarian cancer, and its high mortality rate is mainly because the cancer has progressed to the advanced stage when diagnosed [3]. However, the pathogenesis of ovarian cancer has not yet been elucidated now. Therefore, it is of great significance to clarify the molecular pathogenesis of ovarian cancer and search for efficacious treatment targets for improving the prognosis of ovarian cancer patients.

A circular ribonucleic acid (circRNA) is a highly conservative and stable covalently-closed RNA transcript that is produced by the back-splicing of a

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single precursor messenger RNA (pre-mRNA) with the potential of regulating genes [4]. A growing body of evidence has revealed that circRNAs are closely associated with human diseases, especially tumors, and that they may become potential biomarkers for the features of high abundance and stability [5]. Circular homeodomain interacting protein kinase 3 (circHIPK3), a particularly abundant circRNA, has recently been confirmed to be closely correlated with metabolic disorders and tumorigenesis. CircHIPK3 is notably up-regulated in liver cancer tissues and can promote the proliferation of liver cancer cells [6], but its role in ovarian cancer has not yet been reported at present.

In the present study, the expression of circHIPK3 in the carcinoma tissues and adjacent tissues of ovarian cancer patients was detected, and the expression of circHIPK3 was then inhibited in human ovarian cancer SKOV3 cell lines to observe its effects on the proliferation and apoptosis of SKOV3 cells, so as to provide a basis for the further optimization of treatment schemes for ovarian cancer patients in future.

## Methods

*Tissue specimens*

Carcinoma tissue and adjacent tissue specimens were collected from 66 patients undergoing resection of ovarian cancer in our hospital. After the blood was washed away using normal saline, all the specimens were cut into pieces, placed in Eppendorf (EP) tubes and preserved in a refrigerator at -80°C. This study was approved by the Ethics Committee of Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University. Signed written informed consent was obtained from all participants before the study.

*Human ovarian cancer SKOV3 cell line*

Human ovarian cancer SKOV3 cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cells were cultured with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) containing Roswell Park Memorial Institute (RPMI)1640 complete medium (HyClone, South Logan, UT, USA) in a thermostatic incubator at 37°C, and then sub-cultured once every 2-3 d. Subsequently, the ovarian cancer cells were inoculated into 6-well plates and cultured in the thermostatic incubator for 36 h. Upon reaching the density of 70-80%, the cells in the plates were transfected with circHIPK3 non-sense sequence (NS) or inhibitor according to the instructions of Lipofectmaine™ RNAi MAX transfection reagent (Invitrogen, Carlsbad, CA, USA), and further cultured in the thermostatic incubator at 37°C.

*Detection of circHIPK3 and micro RNA (miR)-7 expressions via reverse transcription-polymerase chain reaction (RT-PCR)*

First, total RNAs were extracted from the carcinoma tissues and adjacent tissues of ovarian cancer patients and human ovarian cancer SKOV3 cell lines using TRIzol (Invitrogen, Carlsbad, CA, USA). Then, the concentration and purity of the RNAs were measured using an ultraviolet spectrophotometer, and absorbance $(A)_{260}/A_{280}$=1.8-2.0 indicated that the concentration and purity of the extracted RNAs were eligible for subsequent experiments. The mRNAs were then reversely transcribed into complementary deoxyribose nucleic acids (cDNAs), and the cDNAs were stored in the refrigerator at -80°C. Afterwards, RT-PCR was performed in the system composed of 2.5 μL of 10× Buffer, 1 μL of cDNAs, 0.5 μL of both 20 μmol/ L forward primers and reverse primers, 10 μL of LightCycler® 480 SYBR Green I Master (2×) and 5.5 μL of $ddH_2O$ (note: the RT-PCR amplification system was the same).Primer sequences used in the study are shown in Table 1.

*RNase R digestion of linear RNAs*

Human ovarian cancer SKOV3 cell lines were divided into digestion group (RNase R group) and non-digestion group (Mock group), and they were prepared with 10× reaction buffer into a reaction system (10 μL) for digestion of linear RNAs. At least 1 U of RNase R should digest 1 μg of RNAs, and the reaction system was stored in the thermostatic incubator at 37°C for 10 min. Finally, the resulting digestion products were extracted, followed by RT-PCR.

*Colony forming assay*

The cells in each group were cultured until the logarithmic growth phase, and digested by 0.25% trypsin into single-cell suspension, and the proportion of single cells should be >95%. Then, the cell suspension was seeded into 6-well plates at an optimal density of 500

**Table 1.** Primer sequences of all indicators in RT-PCR

| *Target gene* | | *Primer sequence* |
|---|---|---|
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Forward | 5'-GACATGCCGCCTGGAGAAAC-3' |
| | Reverse | 5'-AGCCCAGGATGCCCTTTAGT-3' |
| MiR-7 | Forward | 5'-AGCTTCGCTGATGCTGTGTAGC-3' |
| | Reverse | 5'-ACGTAGTGCTCGTAGTATGTGC-3' |
| CircHIPK3 | Forward | 5'-ACGTAGTCGATGTTCAAGCTAG-3' |
| | Reverse | 5'-ACGTAGTCGGCGCCTAGTCGTG-3' |

cells/well. Subsequently, 2 mL of RPMI 1640 complete medium containing 10% FBS was added into each well, and the cell medium was replaced once every 2 d. After 10 d, the cells were fixed using formaldehyde and stained with crystal violet, and the number of the cell colonies in each well was recorded.

*5-ethynyl-2'-deoxyuridine (EdU) staining*

At 48 h after transfection with circHIPK3, the ovarian cancer cells were stained using Click-iT EdU staining kit (Invitrogen, Carlsbad, CA, USA) according to the instructions of the kit. At the end of staining, the cellson each glass slide were photographed in the 3 randomly selected fields of view under a fluorescence microscope. Finally, the EdU-positive cells were counted and quantified.

*Transplantation of ovarian cancer cells for tumorigenesis*

A total of 30 nude mice were randomly assigned into 3 groups: Control group, small interfering (si)-nonsense sequence (NS) group and si-circHIPK3 group, consistent with cell grouping. Matrigel (Corning, Corning, NY, USA) and phosphate buffered saline (PBS) were mixed at 1:1, and the three groups of ovarian cancer cells were re-suspended in the mixture and made single-cell suspension. After being anesthetized by ether, the three groups of nude mice were inoculated with $10^7$ ovarian cancer cells from the three groups, respectively, under the soft skin on the back right forelimb. After 6 weeks, the mice were sacrificed by cervical dislocation, and the tumor masses were removed to measure the size.

*Detection of relevant proteins via Western blotting*

Upon completion of cell stimulation in each group, the medium fluid was discarded, and the resulting medium was rinsed by PBS for three times. Then, the cells in each dish were added with 1,000 μL of cell lysate and thoroughly shaken for 10 min, and those on the dish bottom were scraped fully using a brush and placed in the prepared EP tubes. Subsequently, the collected cells were lysed using an ultrasonic cell cracker for no more than 15 s in total (1-2 s/time). After 15 min of standing, the cells were centrifuged at 12,000 rpm for 0.5 h, and the supernatant was aliquoted into EP tubes. Protein concentration was then measured by the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China) and ultraviolet spectroscopy, and the proteins of all samples were diluted to the same constant concentration, aliquoted and preserved in the refrigerator at -80°C. Followingsodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins in the gels were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), incubated with the PBS-diluted primary antibodies against B-cell lymphoma 2 (Bcl-2) (1:500), Bcl-2 associated X protein (Bax) (1:1,000), glyceraldheyde 3-phosphate dehydrogenase (GAPDH) (1:1,000), and vascular endothelial growth factor (VEGF) (1:500) at 4°C overnight, and then with the goat anti-rabbit secondary antibodies in the dark for 1 h. Finally, the protein bands were scanned by an Odyssey scanner and quantified, with GAPDH as an internal reference.

*Statistics*

All data were analyzed using SPSS 22.0 software (IBM, Armonk, NY, USA), and the measurement data were presented using mean ± standard deviation. Differences between two groups were analyzed by using t-test. Comparison between multiple groups was done using One-way ANOVA, followed by *post hoc* test (Least Significant Difference). $P<0.05$ suggested statistically significant differences.

## Results

*Expression level of circHIPK3 in the carcinoma tissues and normal adjacent tissues of ovarian cancer patients*

According to the RT-PCR results (Figure 1), the expression level of circHIPK3 in the carcinoma tissues of ovarian cancer patients was obviously higher than that in the normal adjacent tissues ($p<0.05$), indicating that circHIPK3 may be correlated with the development and progression of ovarian cancer to a certain degree.

*CircHIPK3 expression verified in ovarian cancer cells and established ovarian cancer cell line with the knockdown of circHIPK3*

Based on the RT-PCR results (Figure 2A), after RNase R digestion, the expression level of the linear HIPK3 mHIPK3 declined obviously in human ovarian cancer cells ($p<0.05$) and the expression level of circHIPK3 was not evidently different from that before the digestion ($p>0.05$). Si-circHIPK3 group exhibited a distinctly lower expression level of circHIPK3 in ovarian cancer cells than Control

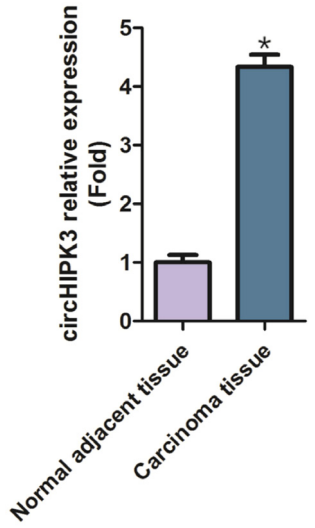


**Figure 1.** Expression level of circHIPK3 in the carcinoma tissues and normal adjacent tissues of ovarian cancer patients: Normal adjacent tissue group: adjacent tissues, and Carcinoma tissue group: carcinoma tissues (*$p<0.05$, compared with Normal adjacent tissue group).

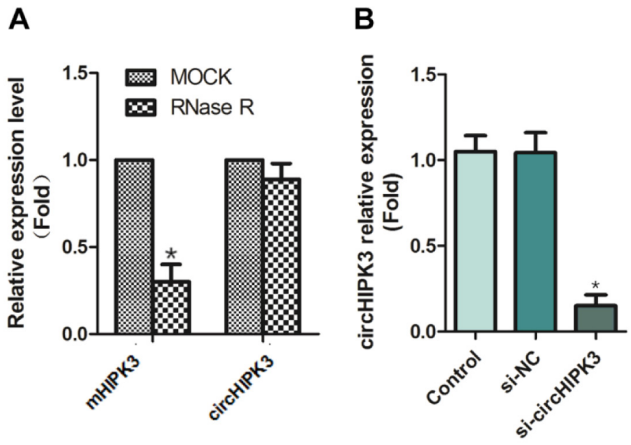


**Figure 2.** Identified circHIPK3 expression in ovarian cancer cells and established ovarian cancer cell line with the knockdown of circHIPK3: mHIPK3: linear HIPK3, and circHIPK3: circular HIPK3 (*p<0.05, compared with Mock group or Control group).

group (p<0.05) (Figure 2B), suggesting that the ovarian cancer cell line with the knockdown of circHIPK3 was successfully established.

*Effect of circHIPK3 knockdown on the colony-forming of ovarian cancer cells*

It was found through the colony-forming assay that the number of colonies formed by ovarian cancer cells was 298.12±0.88, 299.12±1.82 and 45.12±2.98 in Control group, si-NC group and si-circHIPK3 group, respectively (p<0.05) (Figure 3), implying that circHIPK3 has the potential of promoting the clonal proliferation of ovarian cancer cells.

*Effect of circHIPK3 knockdown on the DNA replication of ovarian cancer cells*

As shown by the EdU staining, si-circHIPK3 group had obviously fewer EdU-positive ovarian

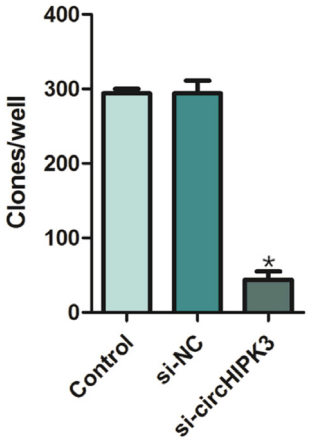


**Figure 3.** Effect of circHIPK3 knockdown on the colony-forming of ovarian cancer cells: Control group: control, si-NS group: circHIPK3 non-sense sequence and si-circHIPK3 group: circHIPK3 inhibitor (*p<0.05, compared with Control group).

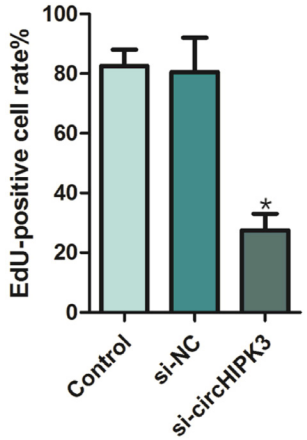


**Figure 4.** Effect of circHIPK3 knockdown on the DNA replication of ovarian cancer cells: Control group: control, si-NS group: circHIPK3 non-sense sequence and si-circHIPK3 group: circHIPK3 inhibitor (*p<0.05, compared with Control group).

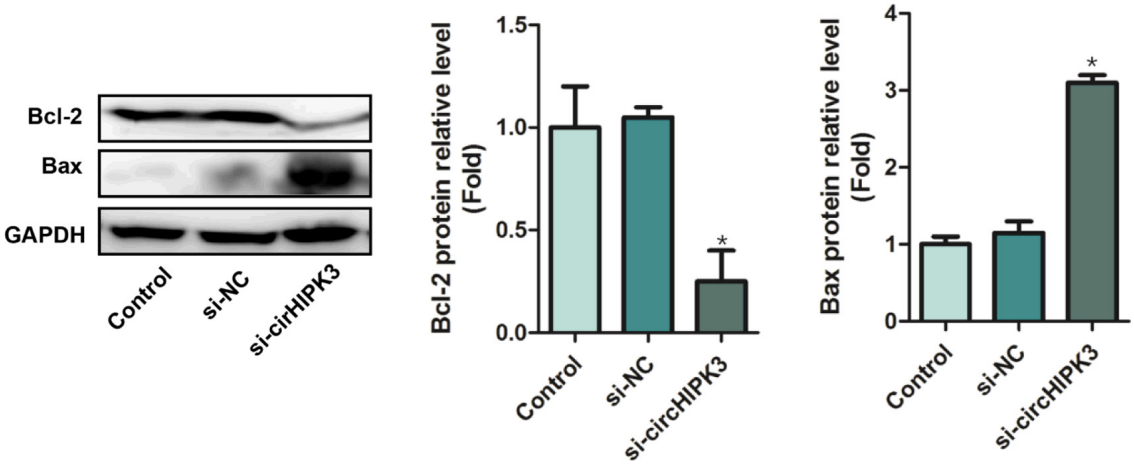


**Figure 5.** Effect of circHIPK3 knockdown on the apoptosis of ovarian cancer cells: Control group: control, si-NS group: circHIPK3 non-sense sequence and si-circHIPK3 group: circHIPK3 inhibitor (*p<0.05, compared with Control group).

cancer cells than Control group (p<0.05) (Figure 4), suggesting that the knockdown of circHIPK3 can restrain the proliferation of ovarian cancer cells.

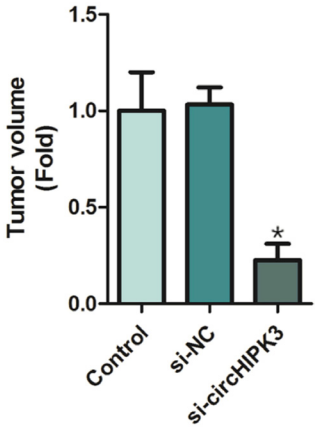


**Figure 6.** Effect of circHIPK3 knockdown on the tumorigenesis of ovarian cancer cells subcutaneously transplanted: Control group: control, si-NS group: circHIPK3 non-sense sequence and si-circHIPK3 group: cancer cells inhibitor (*p<0.05, compared with Control group).

*Effect of circHIPK3 knockdown on the apoptosis of ovarian cancer cells*

According to the Western blotting results (Figure 5), after circHIPK3 was knocked down in ovarian cancer cells, the expression of pro-apoptotic gene Bax obviously rose (p<0.05), but that of anti-apoptotic gene Bcl-2 was distinctly inhibited (p<0.05), indicating that circHIPK3 has a potential inhibitory effect on the apoptosis of ovarian cancer cells.

*Effect of circHIPK3 knockdown on the tumorigenicity of ovarian cancer cells subcutaneously transplanted*

Subcutaneous tumorigenesis assay was further performed in nude mice to observe the tumorigenicity of subcutaneously transplanted allogeneic ovarian cancer cells in the three groups. After 6 weeks, the volume of subcutaneous tumors in nude mice in si-circHIPK3 group was distinctly smaller than that in Control group (Figure 6) (p<0.05), implying that circHIPK3 has the potential of potentiating the *in vitro* tumorigenicity of ovarian cancer cells.

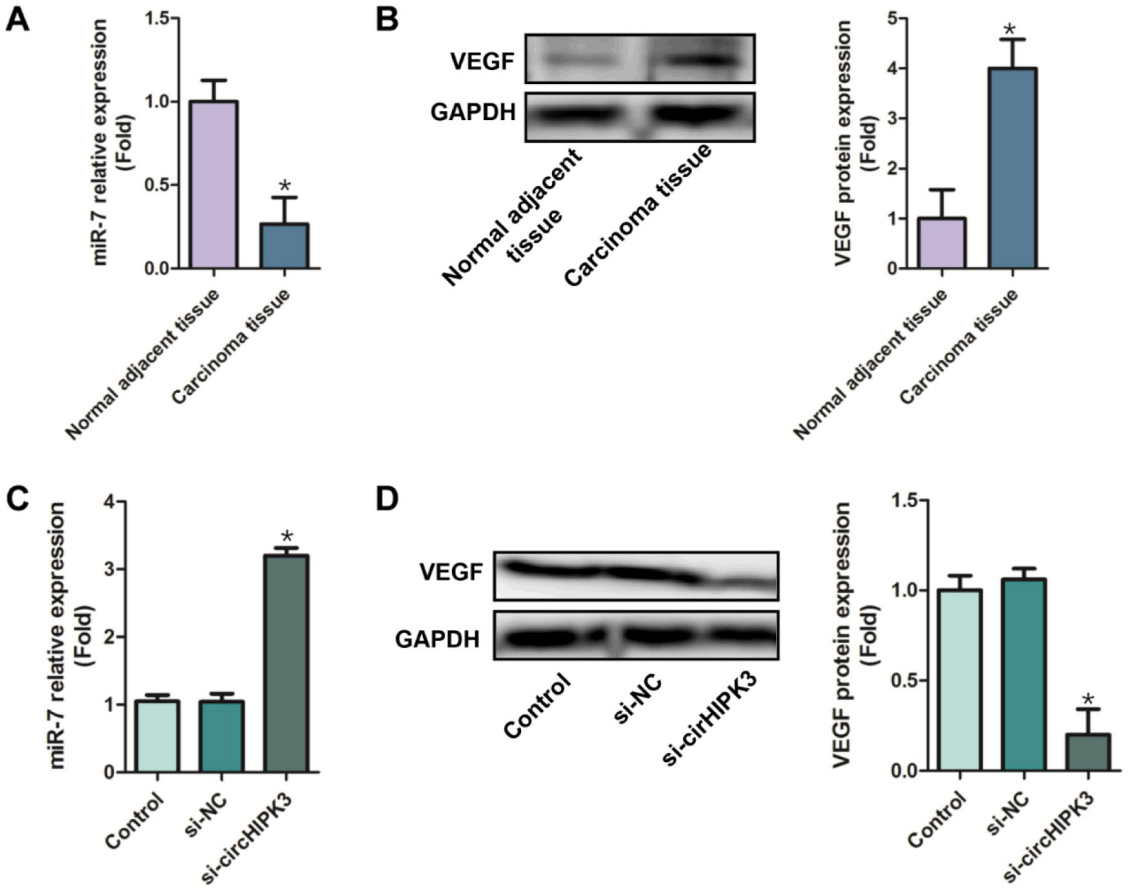


**Figure 7.** Molecular mechanism by which circHIPK3 affected the proliferation and apoptosis of ovarian cancer cells: Normal adjacent tissue group: adjacent tissues, Carcinoma tissue group: carcinoma tissues, Control group: control, si-NS group: circHIPK3 non-sense sequence, and si-circHIPK3 group: circHIPK3 inhibitor (*p<0.05, compared with Control group).

*Molecular mechanism by which circHIPK3 knockdown affected the proliferation and apoptosis of ovarian cancer cells*

Previous studies have demonstrated that circHIPK3 can form a molecular sponge to absorb miR-7, thereby reducing miR-7 content in cells. Moreover, miR-7 can suppress cancers through the targeted inhibition of VEGF. First, the expressions of miR-7 and VEGF in the carcinoma tissues and adjacent tissues of ovarian cancer patients were detected, and it was found that the carcinoma tissues of ovarian cancer patients had an obviously lower expression level of miR-7 ($p<0.05$), but a distinctly higher protein expression level of VEGF ($p<0.05$) than the adjacent tissues (Figure 7A-7B). Then, the *in vitro* cell assay results revealed that the expression of miR-7 was obviously raised ($p<0.05$), but the expression of VEGF was inhibited ($p<0.05$) in ovarian cancer cells after the knockdown of circHIPK3 (Figure 7C-7D).

## Discussion

Ovarian cancer is one of the most aggressive gynecologic cancers worldwide, and about 30-40% of patients with high-grade serous ovarian cancer have a 5-year survival rate of only 30-40% [7]. Despite relatively significant advances in the treatment strategies for ovarian cancer in recent years, the survival and prognosis of ovarian cancer patients have hardly been greatly improved [8]. Most ovarian cancer patients receiving standard treatments will experience recurrence at 6-12 months after the treatments [9]. Therefore, further exploring the pathogenesis of ovarian cancer has an important implication for searching for the key target for the treatment of ovarian cancer.

Growing studies have corroborated that circRNAs play a vital role in the development and progression of cancers and affect their features. CircRNAs can regulate gene expression at the post-transcriptional or transcriptional level, and they mainly exert biological effects by the following mechanisms: 1) CircRNAs serve as the sponges of miRNAs to absorb the free miRNAs in the cytoplasm or nucleus, further affecting the expression of miRNA target genes, 2) circRNAs interact with the Pol II complex to regulate linear RNA transcription and protein expression, and 3) the back-splicing of circRNAs competes with the linear splicing of mRNAs to affect gene expression [10]. Luciferase-based screening assay results suggested that circHIPK3 can sponge 9 miRNAs (miR-124, miR-152, miR-193a, miR-29a, miR-29b, miR-338, miR-379, miR-584 and miR-654), and that circHIPK3 has 18 potential binding sites for miRNAs [11]. Besides, circHIPK3can serve as a sponge to absorb miR-7, thereby promoting the growth and metastasis of colorectal cancer [12]. It can also sponge miR-124 and regulate AQP3 expression to modulate the proliferation and migration of liver cancer cells [13]. In this study, it was found that the expression level of circHIPK3 obviously rose in the carcinoma tissues of ovarian cancer patients, and that the inhibition of circHIPK3 expression by siRNA transfection evidently repressed the proliferation, and promoted the apoptosis of ovarian cancer cells. Furthermore, the results of the present study revealed that the knockdown of circHIPK3 distinctly up-regulated the expression level of miR-7 in ovarian cancer cells, indicating that circHIPK3 may serve as a sponge of miR-7 to induce ovarian cancer. According to previous studies, the inhibition of miR-7 can up-regulate the expression of VEGF by many mechanisms [14,15], and the overexpression of VEGF is one of the important mechanisms in promoting the progression of ovarian cancer [16]. Consistent with the above results, the results of the present study showed that the knockdown of circHIPK3 increased the expression of miR-7, thereby inhibiting the expression of VEGF.

Apoptosis, also known as programmed cell death, is mediated by the intrinsic mitochondrial pathway and the extrinsic death receptor pathway. The former is activated by various external factors, such as ultraviolet or gamma radiation, DNA damage, carcinogenic factors and viral infections [17], and it has two major components: the Bcl-2 family and the inhibitors of apoptotic proteins, which can act as the "apoptosis switches" to modulate mitochondrial membrane permeability [18]. The latter is jointly initiated by the cell-surface receptor tumor necrosis factor (TNF) and the cytokine ligands of TNF superfamily proteins [19]. Studies have demonstrated that the endogenous and exogenous apoptosis pathways can work together to induce cell death. Cancer cells can activate anti-apoptotic molecules or inactivate pro-apoptotic molecules to block their own apoptosis pathways, despite these highly conservative apoptosis pathways in cells [20, 21]. Therefore, the aim of the current studies is to restore the apoptosis pathways in cancer cells without affecting normal adjacent cells. In the present study, it was discovered that the knockdown of circHIPK3 obviously repressed the protein expression of Bcl-2, suggesting that circHIPK3 may restrain the endogenous mitochondrial pathway-mediated apoptosis.

## Conclusions

In conclusion, the present study first reveals that circHIPK3 is highly expressed in the carcinoma tissues of ovarian cancer patients and may sponge miR-7 to induce the high expression of VEGF in ovarian cancer cells, ultimately inducing ovarian cancer.

## Conflict of interests

The authors declare no conflict of interests.

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