

ORIGINAL ARTICLE

CircMUC16 activates AKT3 pathway to promote the malignant progression of cervical cancer

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Summary

Purpose: We aimed at examining CircMUC16 expression in human cervical cancer (CCa) tissues and exploring the role of CircMUC16 and AKT3 pathway in the malignant progression of CCa.

Methods: CircMUC16 expression in 52 pairs of CCa samples and paracancer ones collected from CCa patients of the gynecology department in our hospital was studied by quantitative real-time polymerase chain reaction (qPCR) analysis. CCa cells with stable CircMUC16 knockdown were constructed by using Lentivirus transfection. Changes in the biological functions of CCa cells after CircMUC16 knockdown were determined by qPCR, Western blot, transwell and cell wound healing assays. In addition, the impact of CircMUC16 inhibition on the AKT3 pathway and CCa metastasis were further explored in nude mice *in vivo*.

Results: Marked elevation was revealed in CircMUC16 ex-

pression in the collected CCa tissue specimens compared to adjacent ones. Inhibition of CircMUC16 remarkably attenuated the migration and invasiveness of CCa cell lines. Experiments *in vivo* also suggested that knocking down CircMUC16 could inhibit CCa tumorigenesis in nude mice. The expression of AKT3 pathway-related protein PTEN increased while that of AKT3 decreased after CircMUC16 was inhibited. In addition, co-transfection of CircMUC16 knockdown and AKT3 overexpression vectors reversed the inhibitory effect of single transfection of CircMUC16 knockdown vector on the malignant progression of CCa, both *in vitro* and *in vivo*.

Conclusions: CircMUC16 was highly increased in CCa tissues and it may promote the invasion and migration of CCa via controlling the AKT3 signaling pathway.

Key words: CircMUC16, AKT3 pathway, cervical cancer, malignant progression

Introduction

Cervical cancer (CCa) is one of the female malignancies with high mortality and morbidity [1,2]. In recent years, the death rate caused by CCa and the incidence rate in China has increased steadily, especially in rural areas [3-5]. As we know, this cancer is mainly caused by human papillomavirus infection [6,7]. Although CCa vaccine is successful in preventing this disease, the penetration rate of human papilloma virus (HPV) vaccine is low in developing countries and the drug resistance and side effects induced by chemotherapy make the treatment effect of CCa far from satisfactory, which

lead to CCa becoming one of the killers threatening women's life and health [7,8]. Recurrence and metastasis are still serious problems that clinicians must face in the therapy of CCa. It is difficult to timely monitor the dynamic changes of CCa recurrence or metastasis after surgery and achieve accurate individualized treatment [9,10]. In addition, for CCa patients, the efficacy of radiotherapy and chemotherapy is poor, and the clinical improvement of symptoms is not obvious if imaging test suggest recurrence [11]. Therefore, accurate prediction after surgery is particularly essential [9-

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Received: 24/06/2021; Accepted: 05/08/2021

11], and it is necessary to search for specific and sensitive molecular markers to effectively and objectively evaluate and monitor the postoperative efficacy of CCa [12].

Recently, circular RNA (circRNA), as a new endogenous non-coding RNA, has played an essential part in the occurrence and progression of human diseases. After microRNA (miRNA/miR) and long non-coding RNA(lncRNA), circRNAs are expected to become new biomarkers and therapeutic targets [13,14]. CircRNAs are circular transcripts formed by splicing of exons or introns, lacking 5' and 3' ends [14]. The conservatism, abundance and tissue specificity of circRNA enable it to act as a potential special marker molecule in some diseases [15,16]. Studies have demonstrated that circRNA [15,16] can function as a competitive endogenous RNA or miRNA sponge, and can also interact with proteins to be involved in the regulation of gene expression and protein translation [16,17]. CircRNA has the function of regulating mRNA stability in gene expression and transcriptome [17]. Being widely distributed in human tissues, CircMUC16 plays a pivotal role in many physiological processes, such as the regulation of cell volume, acidification of organelles, cell volume stabilization and bone resorption [18,19], thus involving tumorigenesis and development. This study focused on the relationship between CircMUC16 expression and CCa progression.

Although studies on circRNAs have been carried out in a variety of tumors, we found few studies in CCa. At present, only one article reported that CircMUC16 promotes the proliferation and metastasis of human CCa cells through the AKT3 signaling pathway. We aimed to look for target genes of CircMUC16 related to CCa, and to study their biological functions and their correlation with the prognosis of CCa patients. We characterized a novel axis involving CircMUC16, AKT3 signaling pathway, and CCa cell metastasis that governs the progression of CCa.

Methods

Patients and cervical carcinoma samples

52 cases of CCa tissues and paracancer ones removed from CCa patients in our hospital were collected. No patient was treated with radiotherapy and chemotherapy before surgery. The collected specimens were pathologically diagnosed and staged, and were stored in the RNA specimen preservation solution within 5 min of excision to prevent RNA degradation. CCa pathological classification and staging standards were implemented in accordance with the International Union Against Cancer (UICC) CCa staging standards. This study was approved by the Ethics Committee of Ningbo Yinzhou People's Hospital.

Signed informed consent forms were obtained from all participants before the study entry.

Cell lines and reagents

CCa cell lines (CaSKi, HeLa, HCC94 and C33-A) and normal human cervical epithelial cells (HaCaT) provided by American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS)(Life Technologies, Gaithersburg, MD, USA) in an incubator at 37°C with 5% CO₂.

Transfection

Lipofectamine reagent was mixed with CircMUC16 knockdown sequence (GenePharma, Shanghai, China) and then added into cells when cell density reached 30-50% confluence. 48 h later, cells were collected for subsequent analysis.

Transwell assay

50µL of Matrigel was spread (Matrigel:culture medium= 1: 8, no Matrigel for migration experiments) at the bottom of the transwell chamber. Cells were prepared into cell suspensions 24 h after transfection and seeded in the upper transwell chamber (10,000 cells/well) supplemented with serum-free medium, and then 10% FBS was added to the lower compartment. The migrated cells were counted under a microscope and observed after staining with crystal violet.

Cell wound healing assay

After 48 h, cells were resuspended in medium without FBS to adjust the density to 5×10⁵ cells/mL. After scratch using a pipette tip, cells were rinsed gently with phosphate buffered saline (PBS) for 2-3 times and observed again after incubation in low-concentration serum medium for 24h.

Quantitative real-time polymerase chain reaction (qPCR)

Real-time fluorescence quantitative PCR method was used to detect mRNA levels of CircMUC16, AKT3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in CCa tissues and cells. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the tissues and cells to extract total RNA. Real-time PCR was performed according to the instructions of SYBR® Premix Ex Taq™ (TaKaRa, Tokyo, Japan) kit, with GAPDH as internal reference. Primers used in the qPCR reaction: CircMUC16: forward: 5'-CTGCTCAG-GCCTGTGTTC-3', reverse: 5'-GGGGCCAGCTCTTCA-3'; AKT3: forward: 5'-TTTTCTCTATTATTTGGGCTGAGTC-3', reverse: 5'-CCCCTTTCTGAACCCAACC-3'; GAPDH: forward: 5'-CCTGGCACCCAGACAAT-3', reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western blot

Transfected cells were lysed using cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 × g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) protein assay (Beyotime, Shanghai, China). Immunoblotting was carried out

using primary antibodies against AKT and PTEN (Santa Cruz, Santa Cruz, CA, USA) and the horseradish peroxidase-labeled secondary antibody (Genscript, Nanjing, China). The intensity of protein image was determined using alpha SP image analysis software.

In vivo xenograft model

28-week old male nude mice were purchased from the hospital animal center. CCa cell lines were injected subcutaneously into the axilla of mice. The tumor size was monitored every 5 days and then the mice were sacrificed after 5 weeks. The volume of all samples was calculated using the following formula: tumor volume = (width $2 \times$ length) / 2.

Statistics

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for statistical analyses. Categorical variables were analyzed using χ^2 test or Fisher's exact probability method. Measurement data were compared using t-test and presented as mean \pm standard deviation. $P < 0.05$ was considered statistically significant.

Results

CircMUC16 is highly expressed both in vivo and in vitro

QPCR results showed that CircMUC16 expression in CCa tissue specimens or CCa cell lines was remarkably increased compared with adjacent ones or normal cervical cells HaCaT (Figure 1A, 1B). The above results suggest that CircMUC16 may serve as a new marker to predict the prognosis of CCa.

CircMUC16 knockdown suppresses the invasiveness and migration capacity of CCa cells

To test the impact of CircMUC16 on CCa cell functions, we constructed CircMUC16 knockdown

models in HeLa and HCC94 cells, respectively, and verified the transfection efficiency through qPCR analysis (Figure 2A). We found that knockdown markedly attenuated the ability of CCa cells to migrate and crawl, measured by transwell assay (Figure 2B) and cell wound healing experiment (Figure 2C). Consistently, experiments in nude mice proved that injection of CircMUC16 knockdown vector remarkably reduced tumor volume (Figure 2D) and weight (Figure 2E).

CircMUC16 regulates AKT3 signaling pathway

To further explore through which CircMUC16 regulates AKT3 to promote CCa malignant progression, we first examined the protein expression of AKT by Western blot analysis. Figure 3A shows that transfection of sh-CircMUC16 markedly down-regulated the protein expression levels of AKT and its downstream PTEN. QPCR detection revealed that AKT mRNA expression in CCa tissue samples was higher than that in control groups (Figure 3B). Consistently, *in vitro* experiments in cell lines also showed that AKT3 was remarkably overexpressed in CCa cell lines in comparison to that in HaCaT cells (Figure 3C). Subsequently, we transfected AKT3 overexpression vector into CCa cell lines HeLa and HCC94, and verified the overexpression efficiency by qPCR experiment (Figure 3D).

Overexpression of AKT3 reverses the inhibitory effect of CircMUC16 knockdown on malignant progression of CCa cells

To further explore the mutual regulation of CircMUC16 and AKT3, we simultaneously transfected CircMUC16 knockdown vector and AKT3 overexpression vector into HeLa and HCC94 cell lines, and determined by Western blot that over-

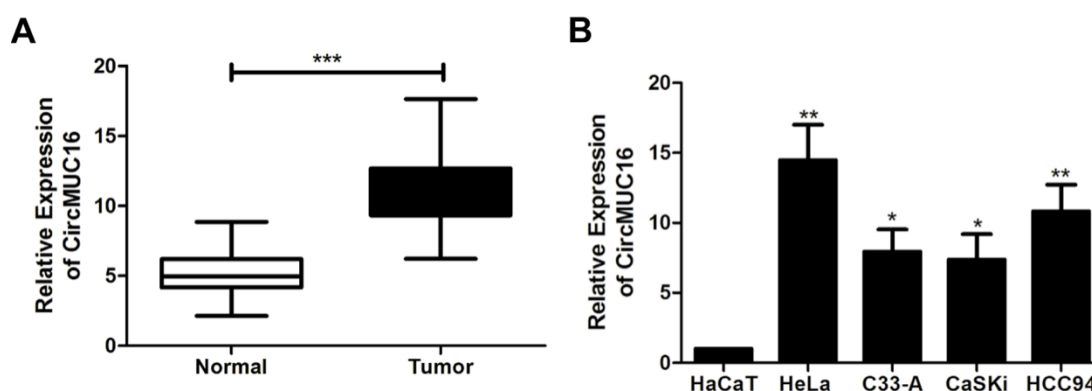


Figure 1. CircMUC16 is highly expressed in cervical cancer tissues and cell lines. **A:** qRT-PCR was used to detect the difference in the expression of CircMUC16 in cervical cancer tumor tissue and adjacent non-tumor tissue. **B:** qRT-PCR was used to detect the expression level of CircMUC16 in cervical cancer cell lines. Data are average \pm SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

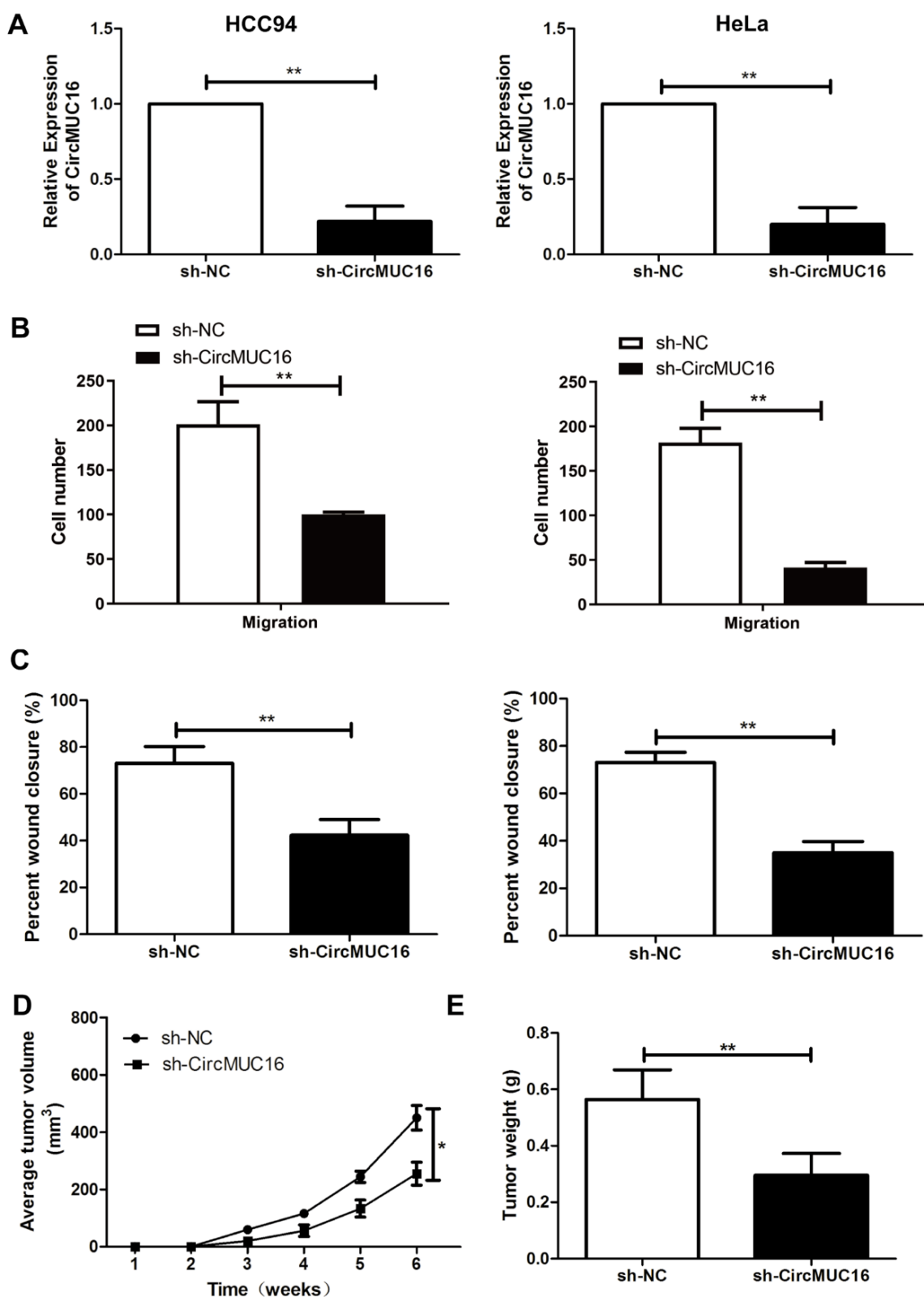


Figure 2. Silencing CircMUC16 can inhibit the proliferation, invasion and migration of cervical cancer cells. **A:** qRT-PCR verified the expression efficiency of CircMUC16 after transfection of CircMUC16 knockdown vector in cervical cancer HeLa and HCC94 cell lines. **B:** Transwell migration test was used to detect the ability of cervical cancer cells to migrate after transfection of CircMUC16 knockdown vector in cervical cancer HeLa and HCC94 cell lines. **C:** Cell wound healing test was used to detect the ability of cervical cancer cells to crawl after transfection of CircMUC16 knockdown vector in HeLa and HCC94 cell lines. **D:** The tumor volume was calculated in different nude mouse groups after injection of cervical cancer HeLa cells transfected with CircMUC16 knockdown vector and the growth curve is depicted. **E:** The tumor weight was calculated in different nude mouse groups after injection of cervical cancer HeLa cells transfected with CircMUC16 knockdown vector. Data are average \pm SD, * p <0.05, ** p <0.01.

expression of AKT3 upregulated the reduced AKT3 expression induced by CircMUC16 knockdown vector (Figure 4A). Meanwhile, knocking down FCMR counteracted the suppressing effect of CircMUC16 downregulation on the migration and crawling ability of CCa cell lines (Figure 4B,4C) and reversed the reduction in the crawling number of transcranial CCa cells in the transwell chamber induced by knockdown of TGIF2 (Figure 4C). Meanwhile, *in vivo* analysis also proved that overexpression of AKT3 could reverse the inhibitory effect of CircMUC16 knockdown vector on the volume (Figure 4D) and weight (Figure 4E) of tumor-forming tissues in nude mice.

Discussion

Cervical cancer, as one of the major malignancies threatening the female reproductive system, has become the second leading cause of cancer death in women [1-4]. In recent years, although the widespread application of HPV DNA and TCT combined screening technology greatly improved the detection rate of CCa, this cancer is still an important cause threatening the survival of women [5,6]. The reason is that we have not fully understood the gene expression and molecular regula-

tion mechanism in the development of CCa, and we have not identified stable molecular markers for the clinical evaluation of CCa patients [9,10]. Therefore, it is necessary to search for specific and sensitive markers to predict the progression of CCa, so as to guide clinical diagnosis and treatment and achieve precise and individualized therapy [10-12].

Recently, circRNA, with a circular structure composed of a special splicing of mRNA precursor and a ring connection between the head and tail of exon sequences, has attracted extensive attention of researchers [13,14]. Meanwhile, in addition to the special circular structure, the biological characteristics of circRNA are also remarkably different from linear RNA [14,15]. circRNAs are not easily degraded due to their stability and spatiotemporal specificity endowed by the special ring structure, and can interact with related miRNA and mRNA to regulate the progress of disease [15,16]. Currently, more and more circRNAs have been discovered to be involved in disease progression [16], among which, CircMUC16 may play an essential part in the development of tumors; however, the specific role of CircMUC16 in CCa still remains unclear [17,18]. The present study demonstrates that CircMUC16 expression in CCa tissues was remarkably higher than that in paracancer normal ones, sug-

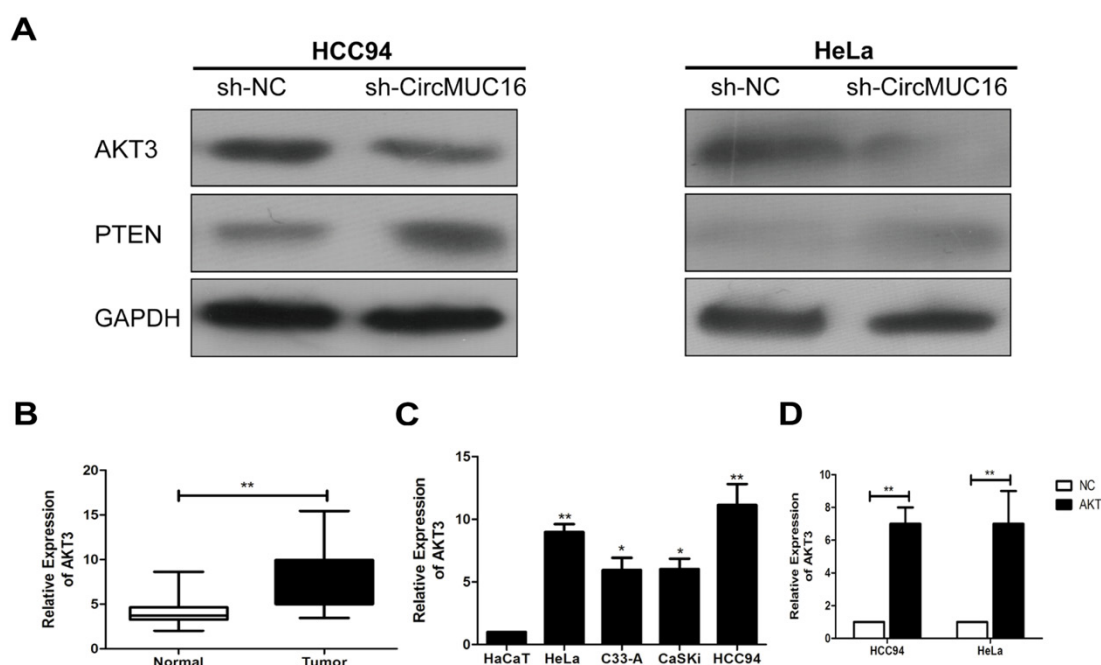


Figure 3. CircMUC16 regulates the AKT3 pathway. **A:** Western blot verified the expression of the AKT3 pathway-related protein AKT3 and PTEN after transfection of CircMUC16 knockdown vector in cervical cancer HeLa and HCC94 cell lines. **B:** qRT-PCR was used to detect the expression of AKT3 in cervical cancer tumor tissues and non-tumor tissues adjacent to cancer. **C:** qRT-PCR was used to detect the expression of AKT3 in cervical cancer cell lines. **D:** Western blot verified the expression of AKT3 after transfection of AKT3 overexpression vector in cervical cancer HeLa and HCC94 cell lines. Data are average \pm SD, * p <0.05, ** p <0.01.

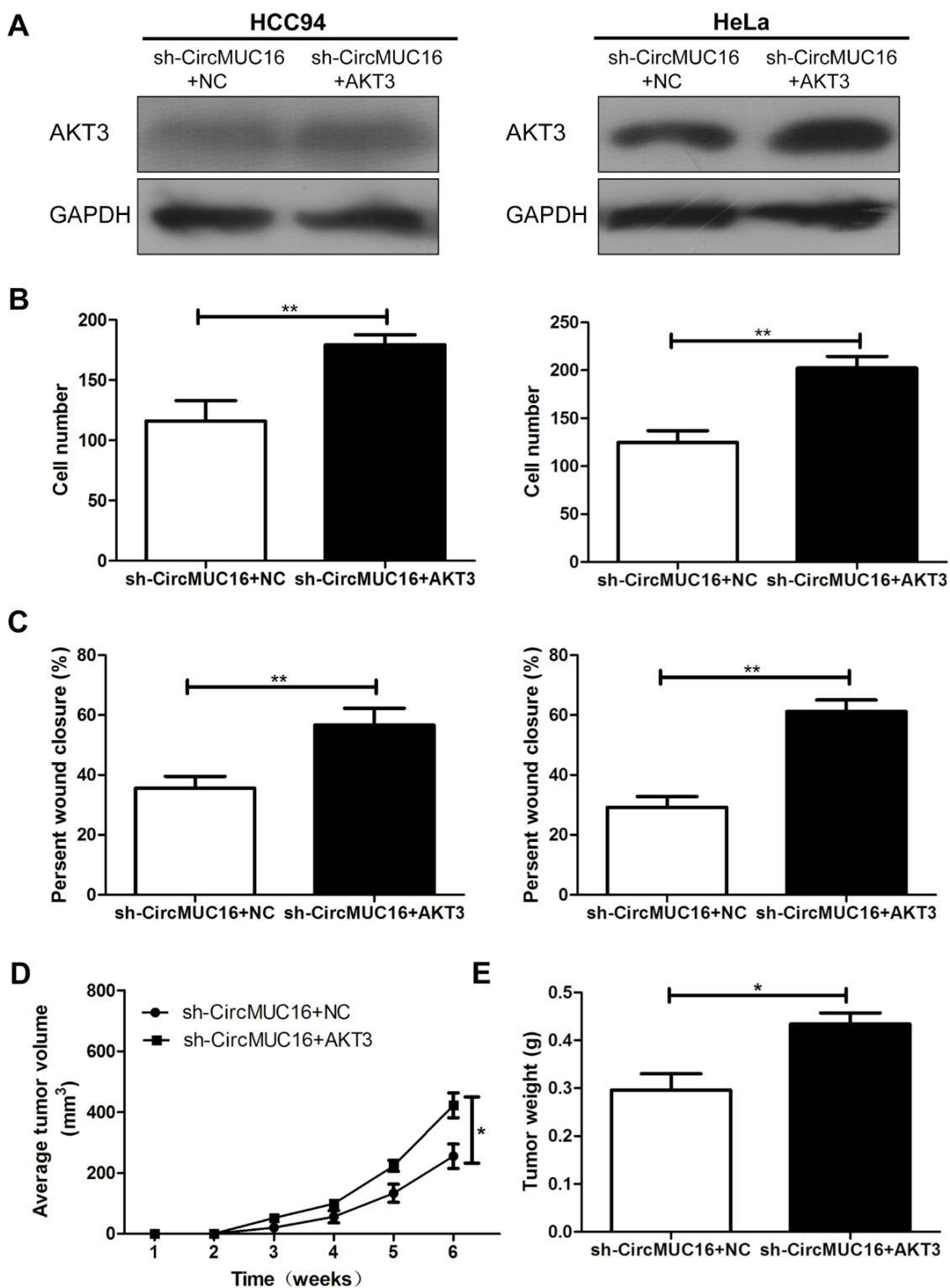


Figure 4. The mutual regulation of CircMUC16 and AKT3 promotes the malignant progression of cervical cancer cells. **A:** Western blot detected the expression level of AKT3 after co-transfection of CircMUC16 knockdown and AKT3 overexpression vector in cervical cancer HeLa and HCC94 cell lines. **B:** Transwell migration assay was used to detect the migration ability of cervical cancer cell after co-transfection of CircMUC16 knockdown and AKT3 overexpression vectors in cervical cancer HeLa and HCC94 cell lines. **C:** Cell wound healing assay was used to detect the crawling ability of cervical cancer cells after co-transfection of CircMUC16 knockdown and AKT3 overexpression vectors in cervical cancer HeLa and HCC94 cell lines. **D:** Growth curves of tumor volume of nude mice was depicted after injection of cells transfected with CircMUC16 knockdown vector or co-transfected with CircMUC16 knockdown and AKT3 overexpression vector. **E:** Comparison of tumor weight of different nude mice after injection of cells transfected with CircMUC16 knockdown vector or co-transfected with CircMUC16 knockdown and AKT3 overexpression vector. Data are average \pm SD, * p <0.05, ** p <0.01.

gesting that CircMUC16 serves as an oncogene in CCa. We then found that knockdown of CircMUC16 inhibited the migration, invasion and tumorigenesis of CCa, but the specific molecular mechanism remains elusive.

CircRNAs directly regulate other RNAs through base complementary pairing and serve as templates for translation to guide protein synthesis [16]. Therefore, it is essential to understand the role of circRNAs in malignant tumors and to explore the underlying mechanism [15]. To clarify the biological function of CircMUC16, we performed bioinformatics analysis and predicted that CircMUC16 may interact with AKT3 signaling pathway. The PI3K/AKT signaling pathway is involved in regulating multiple cellular processes, and overactivation of this pathway is often found in human malignancies and plays a key role in cancer progression [19]. Currently, literature has suggested that AKT3 is a promising new biomarker in the PI3K/AKT signaling pathway, and inhibition of its expression may help develop new therapeutic strategies to suppress the malignant progression of CCa [20,21]. We verified an increase in the protein expression of PTEN

and a reduction in that of AKT3 in CCa cells that were caused by knockdown of CircMUC16. Finally, we proved that overexpression of AKT3 in CCa cell lines could reverse the inhibitory effect of knocking down CircMUC16 on the tumorigenesis ability of CCa cells, further suggesting that CircMUC16 may promote CCa progression by regulating the AKT3 pathway. Therefore, further research is needed to screen out several differentially expressed circRNAs in CCa tissues, as new molecular markers for the comprehensive evaluation of the efficacy and prognosis of CCa.

Conclusions

In summary, CircMUC16 was highly expressed in CCa tissues and cell lines. In addition, CircMUC16 may promote the invasion and migration of CCa through regulating the AKT3 signaling pathway.

Conflict of interests

The authors declare no conflict of interests.

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