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CircRNA ATF6 promotes ovarian cancer cell progression by activating PTEN/mTOR signaling pathway

Lieting MA[1]; Miaoling LI[2,*]; Xinglong ZHENG[3]

[1] Department of Laboratory Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, China
[2] Department of Radiology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, China
[3] Department of Cardiovascular Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, 710061, China

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Abstract: Ovarian cancer is a malignant cancer type and affects women's lives in the world. Circular RNAs (circRNAs) have been involved with the progression of cancers. In our study, we are going to explore the functions of circATF6 in ovarian cancer. The qRT-PCR assay was used to detect expressions of genes. Actinomycin D and RNase R treatment were implemented to verify the circular RNA character of circATF6. Besides, Cell proliferation was assessed by colony formation assay and EdU assay. Silenced circATF6 could reduce the proliferation of ovarian cancer cells. In addition, inhibited circATF6 could promote the cell apoptosis and inhibit related proteins in PTEN/mTOR signaling pathway in ovarian cancer. In conclusion, CircRNA ATF6 promotes ovarian cancer cell progression by activating PTEN/mTOR signaling pathway.

Introduction

Ovarian cancer occupies 4% in female cancers (Torre *et al.*, 2015), and its incidence ranks second only to cervical cancer and endometrial cancer among women (Torre *et al.*, 2015). Estrogens have been reported to play an important regulatory role in the progression of ovarian cancer, which can promote cell growth and activate the aggressiveness of ovarian cancer (Mungenast and Thalhammer, 2014). Many efforts have been made to improve the treatment for ovarian cancer; nevertheless, the 5-year survival rate has yet not been increased. Therefore, for the discovery of more effective treatments for ovarian cancer patients, the underlying mechanism in ovarian cancer will be scrutinized in this study.

Accumulating studies have proved that non-coding RNAs (ncRNAs) participate in various cancer progression (Wang *et al.*, 2018; Zhao *et al.*, 2018). Circular RNAs (circRNAs) have been studied as a group of ncRNAs which have a covalently closed loop feature and can function as competing endogenous RNAs (ceRNA) to regulate the expression of microRNA (miRNA) (Chen *et al.*, 2018; Shao *et al.*, 2018; Wu *et al.*, 2018). Meanwhile, plenty of circRNAs have been disclosed to be aberrantly expressed in ovarian cancer and affect the development of ovarian cancer (Ahmed *et al.*, 2016). For example, circEPSTI1 was found to increase cell growth and invasion in ovarian cancer by regulating the expression of miR-942 (Xie *et al.*, 2019). Meanwhile, circGFRA1 could enhance ovarian cancer cell growth and inhibit apoptosis by modulating miR-449a (Liu *et al.*, 2019). CircATF6 has been discovered located in Chr1(q23.3) with 82034 in genome length and 1522 in spliced sequence length, which is also called hsa_circ_0015018 that expressed in Helas3 cells (Salzman *et al.*, 2013). Moreover, the best transcript of this gene is ATF6. ATF6 knockdown was proved to decrease apoptosis and increase the production of steroid hormones in mouse granulosa cells through endoplasmic reticulum (ER) stress (Xiong *et al.*, 2017), which revealed that circATF6 might have connections with the process of ovarian cancer. Therefore, in our study, the functions of circATF6 will be examined in ovarian cancer.

Materials and Methods

Cell culture and transfection

Human ovarian cancer cells (SKOV3, Caov3, A2780/CP) and normal human ovarian epithelial cells (IOSE-29) were procured from ATCC and preserved in DMEM with 5% CO_2 at 37°C. Medium supplements included 10% FBS and 1% antibiotics. For transfection, the specific shRNAs and NC-shRNAs for circATF6 were all procured from Genepharma Company (Shanghai, China) called sh-circ-ATF6 and sh-NC. After cell confluence reached 85%, sh-circ-ATF6 and sh-NC were transfected into IOSE-29 cells

*Address correspondence to: Miaoling Li, limiaoling101@163.com; Xinglong Zheng, zhengxinglongmay@126.com
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using Lipofectamine 2000 (Invitrogen, USA). Thereafter, cells were incubated for another 24 h, and expressions of genes were quantified by qRT-PCR.

qRT-PCR assay

Total RNAs were extracted from cell samples using the Trizol method for the cDNA synthesis. Gene expression was detected via qRT-PCR using the SYBR Green PCR Master Mix, calculated using the $2^{-\Delta\Delta Ct}$ method and relative to GAPDH. Conditions of PCR was listed as follows: pre-denaturation, 95°C, 5 min; denaturation, 95°C, 30 s; annealing, 60°C, 30 s; extension, 72°C, 30 s, 40 cycles.

Colony formation

Clonogenic cells were collected after transfection and then seeded into the 6-well culture plates for 14-day cell incubation. Mediums then were wiped out, and 4% paraformaldehyde was added to fixing cells and incubated for 15min at room temperature. After fixing, clones were stained in 0.5% crystal violet and cultured at room temperature for 1 h. Later, staining fluid was removed and rinsed with PBS once. Then, the numbers of clones were calculated. Cloning efficiency = numbers of cloning in plates/initial numbers of cells.

EdU assay

Cells in log phase were seeded into a 96-well plate with 5×10^3 cells per well and incubated for 24 h. EdU assay in cell samples was implemented using the standard method of 5-ethynyl-20-deoxyuridine (EdU) incorporation assay kit (Ribobio, Guangzhou China). Then, 50 μL of 4% paraformaldehyde was added to each well and keep culturing at room temperature for 30 min. After that, each cell was added with 50 μL 2 mg/mL glycine to incubate for 30 min. Later, PBS was applied to rinse cells. After DAPI staining, cell samples were subjected to a fluorescence microscope (Leica, Wetzlar, Germany).

Western blot

Cell protein extracts were prepared and treated with 10% SDS-PAGE gel, then shifted onto the PVDF membranes. The primary antibodies available from Abcam (Cambridge, MA) were diluted at 1:2000, while the HRP-tagged secondary antibodies were diluted at 1:5000. After washing, protein samples were assayed by the enhanced chemiluminescence (ECL) fluorescence test kit (Amersham, Arlington Heights, IL).

Statistical analysis

Bio-triplicates were required for all experiments. Results were shown as the mean ± SD and analyzed by GraphPad Prism V5.0. Student's *t*-test and one-way ANOVA were applied for data analysis, with $p < 0.05$ of statistically significant.

Results

CircRNAs were upregulated in ovarian cancer cells

Some circRNAs have been reported aberrantly regulated in cancer and affected the progression of cancer by modulating the cell biological behaviors, such as cell proliferation, apoptosis, and invasion (Ahmed *et al.*, 2016). We detected the expression of circATF6 in ovarian cancer cells at first. A qRT-PCR assay was implemented to assess the expression of circATF6 in ovarian cancer cells (SKOV3, Caov3, and A2780/CP) and normal human ovarian epithelial cell (IOSE-29) (Fig. 1A). We found circATF6 was highly upregulated in ovarian cancer cells than in normal human ovarian epithelial cells, especially in SKOV3 and Caov3 cells. Then, we verified the inhibition efficiency of circATF6 in SKOV3 and Caov3 cells (Fig. 1B). In conclusion, circRNAs were upregulated in ovarian cancer cells.

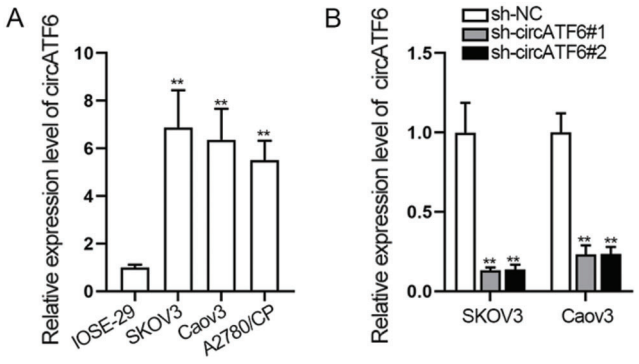

FIGURE 1. CircRNAs was upregulated in ovarian cancer cells. (A) expression of circATF6 was detected in ovarian cancer cells (SKOV3, Caov3 and A2780/CP) and normal human ovarian epithelial cells (IOSE-29) via qRT-PCR assay. (B) inhibition efficiency of circATF6 was examined via qRT-PCR assay in SKOV3 and Caov3 cells.

CircATF6 could function as circular RNA in ovarian cancer cells

Then, we further tested the circular RNA character of circATF6. Treatment of Actinomycin D and RNase R was implemented on SKOV3 and Caov3 cells (Figs. 2A–2B). We found that with the Actinomycin D treatment time increasing, the expression of linear ATF6 was significantly decreased, but the expression of circATF6 has not changed much. Also, RNase R treatment significantly decreased the expression of linear ATF6 and GAPDH. The expression of circATF6 was not reduced. In a word, circATF6 could function as circular RNA in ovarian cancer cells.

Silenced circATF6 could reduce the proliferation of ovarian cancer cells

For testing the function of circATF6 in ovarian cancer cells, functional assays were implemented. Colony formation assay and EdU assay assessed the cell proliferation when circATF6 was depleted (Figs. 3A–3B). Results showed that both the number of colonies and EdU positive cells were reduced by inhibited circATF6. Silenced circATF6 could reduce the proliferation of ovarian cancer cells.

Inhibited circATF6 could promote the cell apoptosis and inhibit PTEN/mTOR signaling pathway in ovarian cancer cells

Moreover, cell apoptosis was identified by qRT-PCR assay. Apoptosis related gene expression (Bal-2, cyclin D1, C-FLIP, caspase3, and caspase7) were tested (Fig. 4A). Results pointed out that Bal-2, cyclin D1, C-FLIP, caspase3, and caspase7 expression levels were all elevated when circATF6 was inhibited, which signified that silenced circATF6 could promote the apoptosis of ovarian cancer cells. PTEN/mTOR

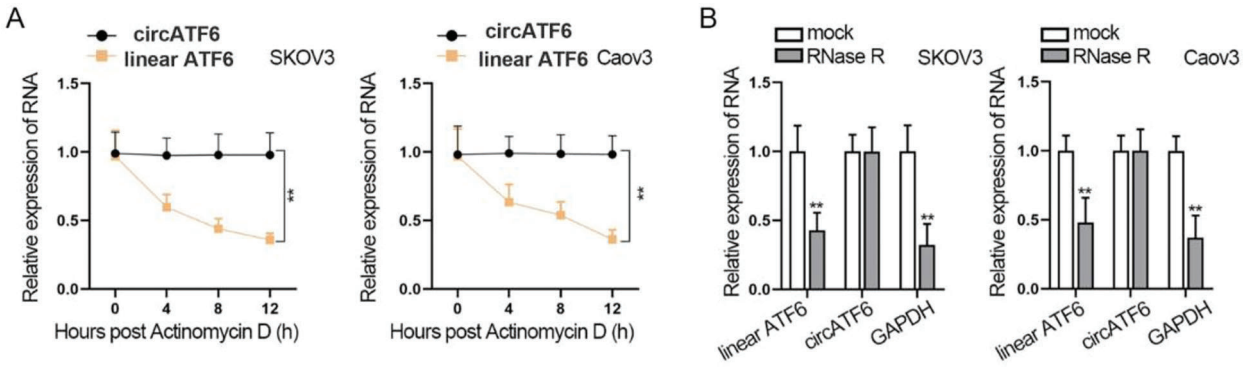

FIGURE 2. CircATF6 could function as circular RNA in ovarian cancer cells. (A-B) Actinomycin D and RNase R treatment was implemented to verify the circular RNA character of circATF6.

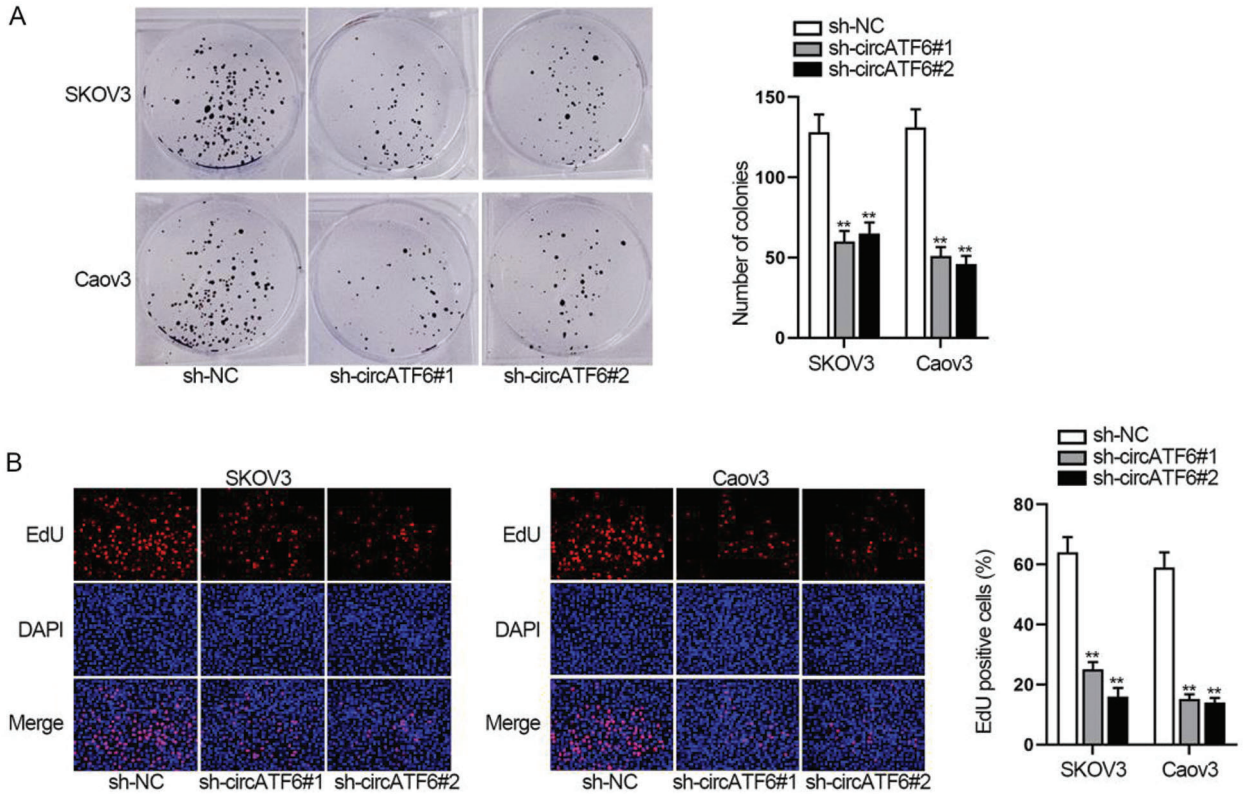

FIGURE 3. Silenced circATF6 could reduce the proliferation of ovarian cancer cells. (A-B) Cell proliferation was assessed by colony formation assay and EdU assay when circATF6 was depleted.

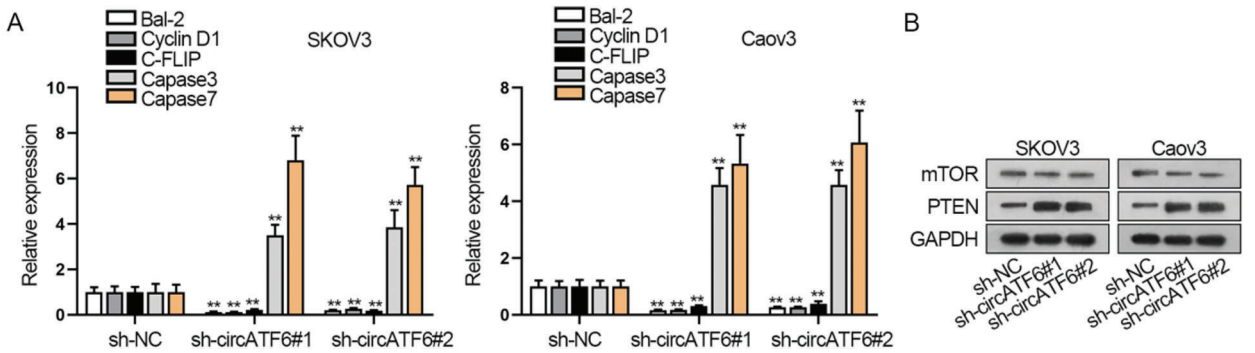

FIGURE 4. Inhibited circATF6 could promote the cell apoptosis and inhibit PTEN/mTOR signaling pathway in ovarian cancer cells. (A) Apoptosis-related gene expression was tested via qRT-PCR assay. (B) Western blot assay was conducted to know about the protein level change of PTEN and mTOR when circATF6 was inhibited.

signaling pathway has been reported as a classical pathway in modulating the progression of cancers (Riquelme *et al*., 2016). Thence, we adopted a western blot assay for the protein level of PTEN and mTOR (Fig. 4B). We found that the mTOR protein level was decreased, and PTEN protein level was elevated by silenced circATF6. In a word, inhibited circATF6 could promote the cell apoptosis and inhibit PTEN/mTOR signaling pathway in ovarian cancer.

Discussion

CircRNAs can act as tumor activator or tumor suppresser in cancers. For instance, CircRNA_102171 can increase the cell growth and migration of papillary thyroid cancer by activating the pathway of β-catenin (Bi *et al*., 2018). Meanwhile, CircRNA-100338 can exacerbate the prognosis of hepatocellular carcinoma by modulating the expression of miR-141-3p and RHEB to regulate mTOR (Huang *et al*., 2019). Also, circRNA_102958 can promote colorectal cancer cell growth and invasion by regulating the miR-585/CDC25B pathway (Li *et al*., 2019). In our study, we firstly identified the expression of circATF6 in ovarian cancer cells via qRT-PCR, and we found that circATF6 was significantly highly expressed in ovarian cancer cells. Then, we further detected the circular character of circATF6 via RNase R and actinomycin D treatment. We found that circATF6 expression was not changed by RNase R and actinomycin D treatment. Moreover, functional assays such as colony formation assay and EdU assay were implemented to detect the cell proliferation of ovarian cancer, and results indicated that cell proliferation was significantly decreased by inhibited circATF6. Meanwhile, the qRT-PCR assay was implemented to verify the cell apoptosis. Apoptosis related genes were tested. Furthermore, we detected the cell apoptosis was increased via silenced circATF6.

PTEN/mTOR signaling pathway has been reported as a classical pathway in modulating the progression of cancers (Riquelme *et al*., 2016). PTEN is known as a tumor suppressor in varied cancers (Lazaridis *et al*., 2019; Sun *et al*., 2018). mTOR has been widely explored in a diversity of cancers, which was confirmed to be a tumor promoter (Li *et al*., 2018). Activation of the PTEN/mTOR signaling pathway, namely, the suppression of PTEN and the upregulation of mTOR can promote cell proliferation and inhibit cell apoptosis in cancers (Kremer *et al*., 2006). In the present study, the protein concentrations of PTEN and mTOR were investigated via western blot assay, and results displayed that PTEN was upregulated while mTOR was decreased, suggesting the inactivation of the PTEN/mTOR signaling pathway when circATF6 was silenced in ovarian cancer cells. Therefore, we concluded that circular RNA ATF6 might induce cell proliferation and inhibits cell apoptosis by activating PTEN/mTOR signaling pathway in ovarian cancer.

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Conflicts of Interest: Authors declare no conflicts of interest in this study.

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