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ORIGINAL ARTICLE

A study of zederone for the inhibition on ovarian cancer cell proliferation through mTOR/p70s6K signalling pathway

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Summary

Purpose: *To investigate the inhibitory effect of Zederone (Zed) on the proliferation of human ovarian cancer cell line SK-OV-3 (SKOV3) and to explore the possible mechanism.*

Methods: *Cell Counting Kit-8 (CCK-8) assay was performed to detect the inhibitory effect of different concentrations of Zed on the proliferation of SKOV3 cells; the effect of Zed on the morphology of SKOV3 cells was observed; flow cytometry was performed to investigate the effect of Zed on the cycle phase distribution of SKOV3 cells; Real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) and western blot were performed to detect the effects of Zed on the expression of mTOR, p70s6K, p-PI3K and p-Akt at mRNA and protein level in SKOV3 cells, respectively.*

Results: *Zed could effectively inhibit the proliferation of SKOV3 cells in vitro and change cell morphology. Flow cytometry indicated that Zed arrested SKOV3 cells at G1 phase. qRT-PCR revealed that Zed downregulated the mRNA levels of mTOR and p70s6K. However, western blot demonstrated that Zed could downregulate the protein expressions of mTOR, p70s6K, phosphorylated mTOR (p-mTOR) and phosphorylated p70 S6 kinase (p-p70s6K) in SKOV3 cells, but had no significant influences on p-PI3K and p-Akt proteins.*

Conclusion: *In conclusion, Zed can significantly inhibit the proliferation of human ovarian cancer SKOV3 cells, and this regulation may be mediated by the inhibition of mTOR/p70s6K signal pathway.*

Key words: *human ovarian cancer cell SKOV3, mTOR/p70s6K, proliferation, zederone*

Introduction

Ovarian cancer is a common genital system malignancy in females. The incidence rate of ovarian cancer is only lower to that of cervical and endometrial cancer, ranking third among all malignant tumors [1]. However, the mortality rate of ovarian cancer is the highest, which seriously affects women's health and life. At present, the administration of chemotherapy in the treatment of ovarian cancer is challenged by serious adverse reactions and drug resistance. Therefore, the development of novel effective anti-cancer compounds with less adverse reactions is always needed [2].

Curcuma zedoary is the dry rhizome of curcuma phaeocaulis Val, Guangxi curcuma or Curcuma wenyujin. Studies have shown that Zedoary extract has liver protection, anti-bacteria, anti-thrombosis, gastrointestinal motility promotion and anti-tumor activities [3,4]. Zederone (Zed) is a main physiologically active ingredient in Curcuma phaeocaulis. Studies have found that Zed can inhibit the proliferation of colon cancer and prostate cancer cells *in vitro* [5-7].

The target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates a variety

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of intracellular signal pathways. mTOR regulates a variety of biological processes such as cell growth, proliferation, apoptosis and autophagy [8]. p70S6 kinase (p70S6K) is the downstream substrate of the mTOR protein, and phosphorylated mTOR (p-mTOR) can further phosphorylate p70S6K to produce phosphorylated p70S6K (p-p70S6K) [9]. Studies have found that mTOR/p70S6K signal pathway is closely correlated to the occurrence and development of a variety of malignant tumors [10].

Our previous study and screening showed that Zed had a strong inhibitory effect on human ovarian cancer SKOV3 cells [11]. This study aimed to investigate the inhibitory effects of Zed on the proliferation of cells of human ovarian cancer line SKOV3. Cell Counting Kit-8 (CCK-8) and flow cytometry were used to detect the effect of Zed on the proliferation and cell cycle of SKOV3 cells. To further explore the possible mechanism of the function of Zed, the effects of Zed on the mRNA and protein expressions of genes related to mTOR/p70s6K signal pathway were detected by qRT-PCR and western blot. Our study provided basis for the application of Zed in the treatment of ovarian cancer.

Methods

Materials

Human ovarian cancer cell strain SK-OV-3 (SKOV3) and Human normal ovarian epithelial cell strain IOSE80 (Cell Bank of Chinese Academy of Sciences, Shanghai, China); Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA,USA); Zed, dimethyl sulfoxide (DMSO), Dimethyl sulfoxide (DMSO) (Sigma, Louis, MO, USA); cell counting kit-8 (CCK-8) detection kit; cell cycle detection kit (Beyotime Institute of Biotechnology, Nantong City, China); TRIzol, reverse transcription kit, qRT-PCR kit (Invitrogen, Carlsbad, CA, USA), primer synthesis (TaKaRa, Dalian, China); mTOR, p-mTOR, p70s6K, p-p70s6K, p-PI3K, p-Akt β-actin and horse radish peroxidase (HRP) labeled secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA).

Cell culture

SKOV3 and IOSE80 cells were cultured in RPMI-1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal calf serum (FCS) and cultured in a cell incubator with 5% CO_2 at 37°C. Cells were digested by trypsin when 90% of the bottom of the bottle was covered, followed by passage.

Detection of the effect of Zen on SKOV3 cell proliferation by CCK-8 method

Zed was dissolved in dimethylsulfoxide (DMSO) to make a stock solution (80 mg/mL). After the trypsin digestion, cell density was adjusted to be 1×10^5/mL and cells were inoculated on 96-well plates with 100 μL in each well. After 24 h of regular culture, Zed culture solution was added to a final concentration of 0, 5, 10, 20, 40, 80 μg/mL, respectively. Another well with same amount of DMSO was set as control. After cell culture for 24 h, 20μL CCK8 solution was added into each well, followed by incubation for 2 h in an incubator. The optical density (OD) value at 450 nm of each well was inspected with a microplate reader. The inhibition ratio of Zed on cells was calculated according to the following equation: Inhibition ratio (%)= [(*OD* value of experimental group - *OD* value of normal control group)/*OD* value of normal control group] ×100%.

Observation of the effect of Zed on SKOV3 cell morphology by microscope

After trypsin digestion, cell density was adjusted to 2×10^5/mL and cells were inoculated on 6-well plates with 100 μL per well. After regular culture for 24 h, cells were divided into the normal control group (Control, with same amount of DMSO) and the experimental group. The control group was cultured in routine culture medium, Zed was added into the experimental group to a final concentration of 10, 20 and 40 μg/mL, respectively. After culturing for 24 h, morphological cell change in each group was observed and photographed under inverted microscope.

Detection of the effect of Zed on the SKOV3 cell cycle by cell cycle kit

According to the grouping and treatment methods previously described, cells were cultured for 24 h and flow cytometry was applied to detect the effect of Zed on the SKOV3 cell cycle according to the instructions of cell cycle kit. After trypsin digestion, cells were collected after centrifugation. Then, 2 mL precooled 70% ethyl alcohol was added, followed by fixation in a refrigerator at 4°C overnight. Then, ethyl alcohol was removed and 25 μL propidium iodide (PI) solution, 10 μL RNaseA

Table 1. qRT-PCR primer sequences

Gene name	*Primer name*	*Primer sequence*
mTOR	Forward primer	5'-CGCTGTCATCCCTTTATCG-3'
	Reverse primer	5'-ATGCTCAAACACCTCCACC-3'
p70S6K	Forward primer	5'-TACTTCGGGTACTTGGTAA-3'
	Reverse primer	5'-GATGAAGGGATGCTTTACT-3'
β-actin	Forward primer	5'-ACCAACTGGGACGACATGGAGAAAATC-3'
	Reverse primer	5'-GTAGCCGCGCTCGGTGAGGATCTTCAT-3'

solution and 500 μL staining buffer were added. After incubation for 30 min in an incubator at 37°C, cells were rinsed with phosphate buffered saline (PBS) and analyzed using flow cytometry.

Detection of expressions of mTOR and p70s6K mRNA in SKOV3 cells via qRT-PCR

After cell culture for 24 h, cells were digested with trypsin and collected for centrifugation. Total RNA was extracted from cells by TRIzol method. RNA purity was detected by the UV spectrophotometer. RNA samples with the A260/A280 ratio between 1.8 and 2.0 were subjected to reverse transcription synthesize complementary DNA (cDNA) [11]. PCR was conducted according to instructions of the kit. Primer sequences are shown in Table 1 and PCR conditions are as follows: 95°C for 3 min, followed by 30 cycles of 95°C for 3 s, 54°C for 30 s and 72°C for 1 min. T *Ct* value were processed using $2^{-\Delta Ct}$ method.

Detection of the expression of proteins related to mTOR/p70s6K signalling pathway in SKOV3 cells by western blot

After cell culture for 48 h, cells were digested by trypsin and collected by centrifugation. Radio immune precipitation assay (RIPA) lysis buffer was added and incubated on ice for 30 min, followed by centrifugation at 12000g under low temperature. The supernatant was collected and protein concentration was determined by bicinchoninic acid (BCA) protein assay kit. Then, 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with protein loading amount of 60 μg/well. Protein was transferred onto the nitrocellulose membrane after the electrophoresis and blocked using 5% skim milk powder. Then, primary antibodies of mTOR, p-mTOR, p70S6K, p-p70S6K, p-Akt, p-PI3K and β-actin (diluted at 1:1000) were incubated with the membrane at 4°C overnight, followed by incubation with secondary antibody (1:2000) at room temperature for 2 h. The membrane was washed, followed by signal development via electrochemiluminescence (ECL) in the dark. The results were analyzed using Image Lab 4.0.4 software.

Statistics

Data were processed using SPSS 17.0 (International Business Machines Corporation, USA) software. Data were expressed as mean±standard deviation. Unpaired Student's *t*-test was used for comparison between groups. P<0.05 indicated a difference with statistical significance.

Results

The effect of Zed on cell proliferation

As shown in Figure 1, compared with the control group, Zed inhibited the proliferation of SKOV3 cells (p<0.01) in a dose-dependent manner. However, Zed had no significant effect on the proliferation of IOSE80 cells (p>0.05). According to the results of CCK-8, the drug concentration at median lethal dose was used for subsequent experiments (10, 20 and 40μg/mL).

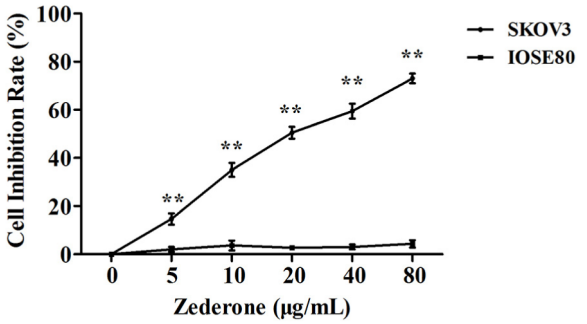

Figure 1. Detection of the proliferation of SKOV3 and IOSE80 cells treated with different concentrations of Zed via MTT. Compared with 0 μg/mL (normal control group), Zed can significantly inhibit the proliferation of SKOV3 cells. **compared with the normal control group, p<0.01.

The effect of Zed on SKOV3 cell morphology

As shown in Figure 2, compared with the normal control group, cell morphology was changed significantly after treatment with Zed treatment for 24h. The number of cells was decreased, and cells were shrinked and wrinkled, and some cells were round and had lost the original normal cell morphology.

The effect of Zed on cycle of SKOV3 cells

The effect of Zed on cell cycle of SKOV3 cells line is shown in Figure 3. Compared with the nor-

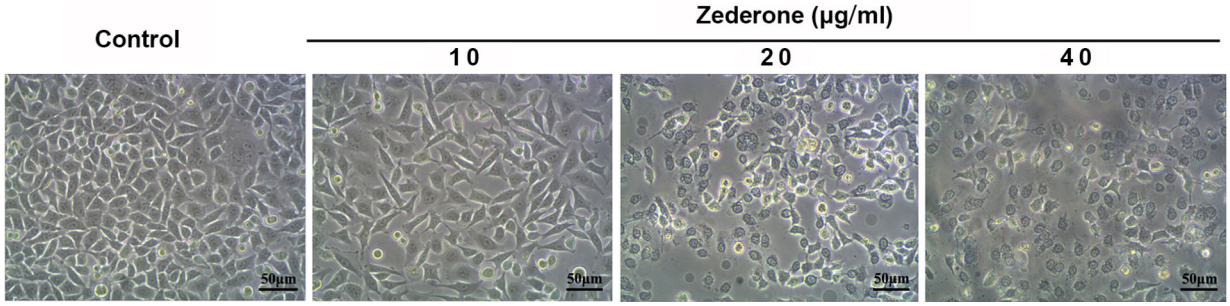

Figure 2. The effect of Zed on the morphology of SKOV3 cells (200×). Cells shrink, and some cells appear to be round and have lost the original normal cell morphology when treated with Zed.

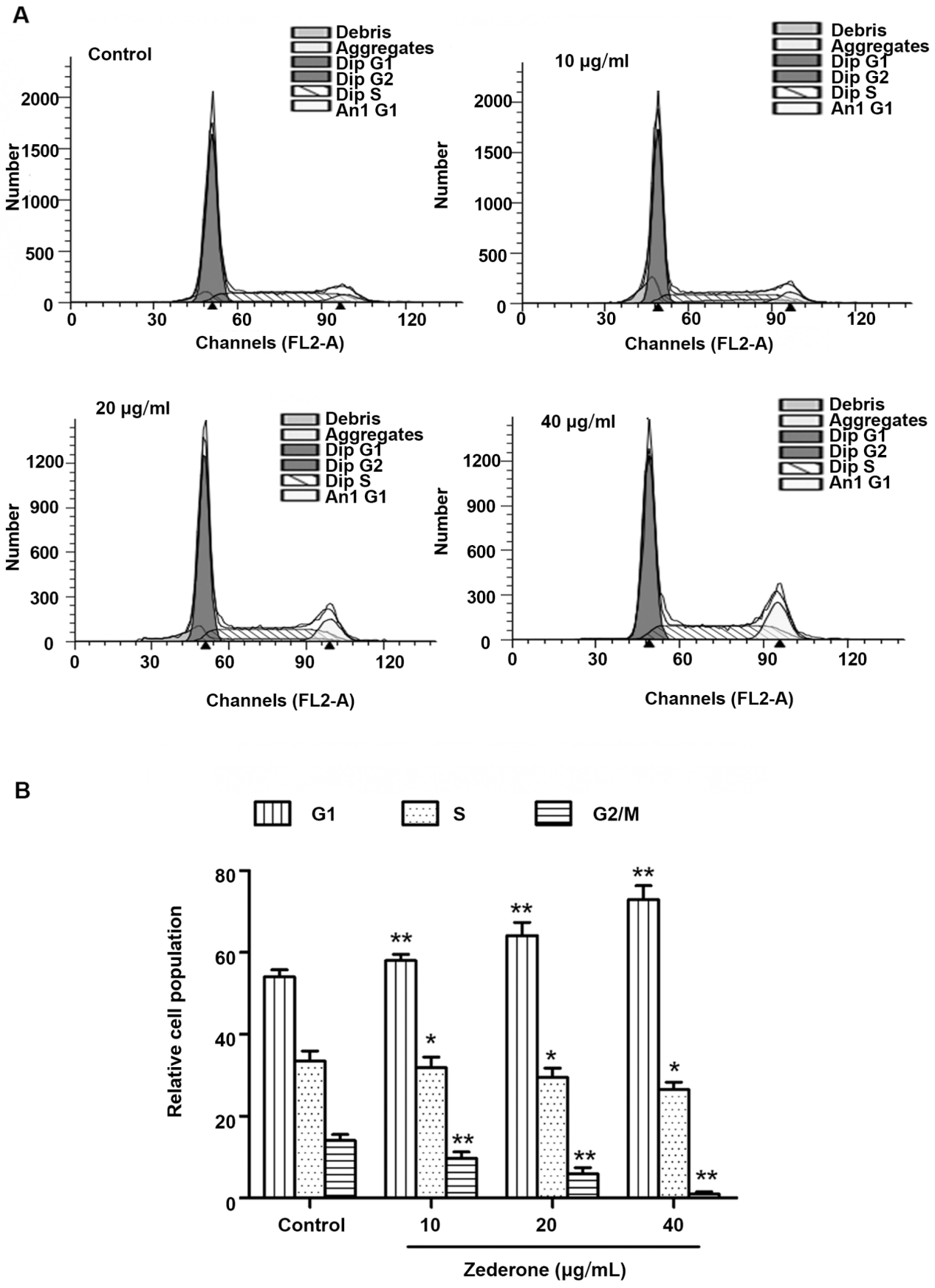

Figure 3. Detection of the effect of Zed with different concentrations on cell cycle of SKOV3 cells via flow cytometry. Zed can arrest SKOV3 cells at G1 phase. **A:** The flow cytometry results of each group of cells; **B:** Statistical chart of the cell proportion in each group. *compared with normal control group, p<0.05; **compared with the normal control group, p<0.01.

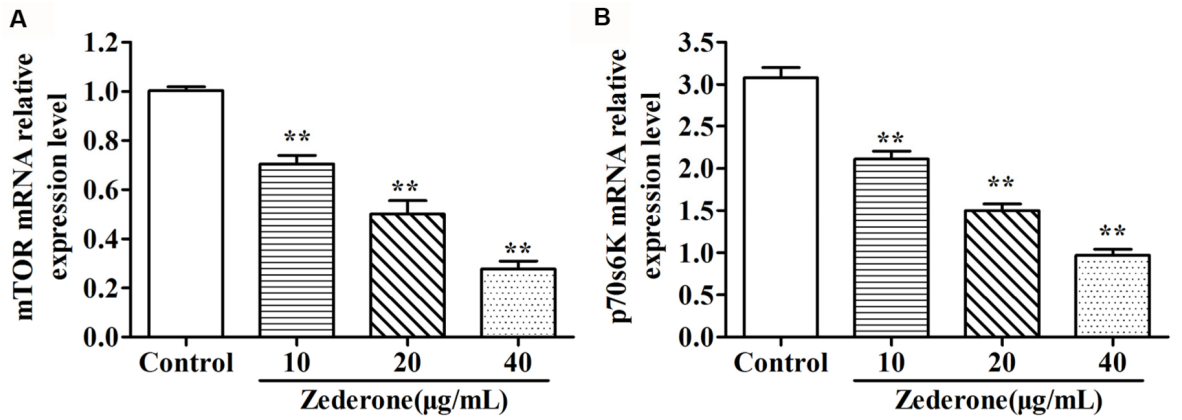

Figure 4. Detection of the effect of Zed on the expression of mTOR and p70s6K mRNA in SKOV3 cells via qRT-PCR. **A:** The expression levels of mTOR mRNA were significantly decreased in Zed groups with different concentrations compared with those in the normal control group. **B:** The expression levels of 570sK mRNA were significantly decreased in Zed group with different concentrations compared with those in the normal control group. Zed can significantly promote the expression of Bax and Caspase 9 mRNA in SKOV3 cells. **compared with the normal control group, p<0.01.

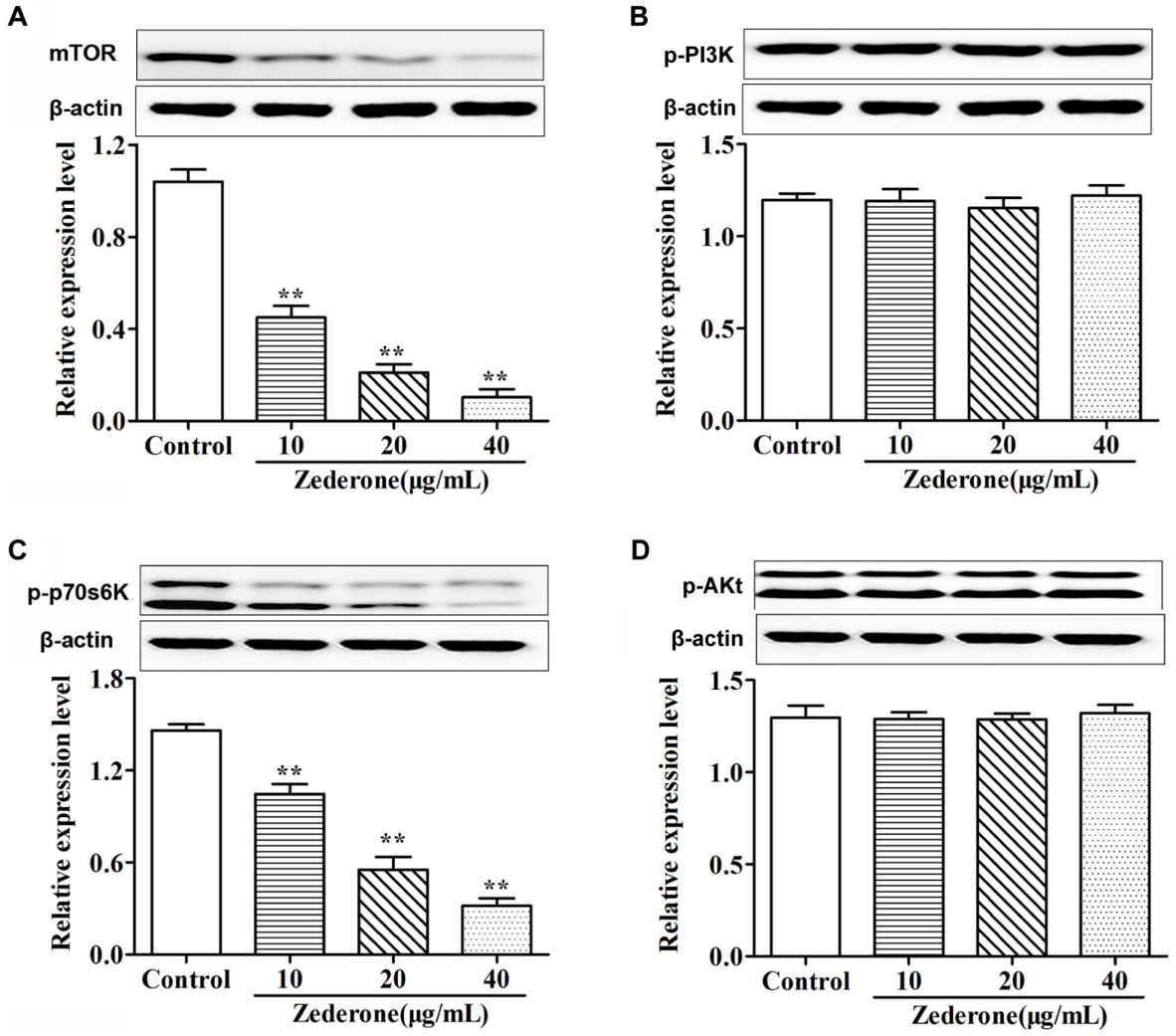

Figure 5. The effect of Zed on the expressions of mTOR, p-p70s6K, p-PI3K and p-Akt protein in SKOV3 cells detected by Western blot. **A:** The expression levels of mTOR were significantly decreased after treatment with Zed (10, 20 and 40μg/mL). **B:** The expression levels of p-PI3K were almost unchanged after treatment with Zed (10, 20 and 40μg/mL). **C:** The expression levels of p-p70s6K were significantly decreased after treatment with Zed (10, 20 and 40μg/mL). **D:** The expression levels of p-Akt were almost unchanged after treatment with Zed (10, 20 and 40μg/mL). **compared with the normal control group, p<0.01.

mal control group, after 24 h action of Zed on SKOV3 cells, the ratio of G1 phase cells in total cells was significantly increased ($p<0.01$), while the ratio of S phase cells and G2/M phase cells was significantly decreased ($p<0.05$). The results showed that Zed could arrest SKOV3 cells at G1 phase, so as to inhibit the cell proliferation.

Effects of Zed on mTOR and p70s6K mRNA expression in SKOV3 cells

As shown in Figure 4, qRT-PCR results showed that expression levels of mTOR and p70s6K mRNA were significantly decreased in Zed groups with different concentrations compared with those in the normal control group ($p<0.01$). With the increase of Zed concentration, the expressions of mTOR and p70s6K mRNA were decreased gradually.

Effects of Zed on the protein expressions of mTOR and p70s6K signal pathways in SKOV3 cells

As shown in Figure 5, western blot results revealed that, compared with the control group, the expression levels of p-mTOR, p-p70s6K, mTOR and p70s6K in Zed-treated SKOV3 cells were significantly decreased ($p<0.01$), but the expression levels of p-PI3K and p-Akt were basically unchanged, indicating that Zed can reduce the expression and phosphorylation of mTOR and p70s6K in SKOV3 cells.

Discussion

Ovarian cancer patients usually have low survival rates because most patients were diagnosed in advanced stages. In order to improve the survival rate of patients, molecular targeted therapies have been developed [13]. Numerous studies have found that mTOR and p70S6K proteins play important roles in the development and progression of ovarian cancer [14].

PI3K/Akt/mTOR signal transduction pathway can promote cell growth, proliferation, differentiation and invasion, inhibit cell apoptosis, regulate immune function and promote angiogenesis [15]. mTOR is a serine/threonine protein kinase with a molecular weight of about 289 kDa and it is a member of PI3K protein family because its C-terminal is highly homologous to the catalytic domain of PI3K. mTOR signal transduction pathway plays an important role in the cell cycle progression and the abnormal regulation of cell cycle progression is closely related to cancer. Therefore, mTOR signal pathway is the critical development and progression of cancer [16]. Murayama et al found that mTOR expression in the cytoplasm is positively correlated with the invasion depth of gastric cancer, lymph node metastasis and tumor staging [17]. In addition, other studies have reported that mTOR is activated in pancreatic cancer cells and mTOR inhibitors can inhibit the proliferation of pancreatic cancer cells [18]. p70S6K is the main downstream target protein of mTOR and the key regulator of mTOR downstream substrate. p70S6K has ribosomal protein kinase activity and can activate S6 protein of ribosomal 40S small subunit to increase the translation efficiency of related genes [19]. p-p70S6K can regulate the growth and proliferation of cells by affecting the synthesis of ribosomes and other related proteins [20]. It was found that the activation of mTOR/p70s6K signal pathway may affect the transcription and translation of key genes in cell cycle and arrest the tumor cells at G1 phase, which in turn disorders cell division and growth processes [21]. Rowinsky et al reported that the expressions of cyclinD/myc and other cell cycle-related proteins are regulated by mTOR/p70s6K signalling pathway [22].

In this study, ovarian cancer SKOV3 and IOSE80 cells were treated with Zed at different concentrations. After 24 h of treatment, various indicators were detected. CCK-8 test results showed that Zed could significantly inhibit the proliferation of SKOV3 cells and change SKOV3 cell morphology significantly, making cell surface shrink and winkle. There was no significant effect on normal human ovarian epithelial IOSE80 cells. Flow cytometry showed that the proportion of G1 phase cells was increased, and the ratios of cells in S phase and G2/M phase were decreased. qRT-PCR results showed that Zed could significantly decrease the expressions of mTOR and p70s6K mRNA in SKOV3 cells in a dose-dependent manner. Western blot results showed that Zed could significantly reduce the expression of p-mTOR, p-p70s6K, mTOR and p70s6K in SKOV3 cells ($p<0.01$), but had no effect on the expression of p-PI3K and p-Akt, indicating that Zed can inhibit the expression and phosphorylation of mTOR and p70s6K protein in SKOV3 cells. Wu et al found that microRNA-145 (miR-145) can inhibit the proliferation of ovarian cancer cells by downregulating the phosphorylation of p70s6K protein [23]. This study found that Zed could downregulate the phosphorylation levels of mTOR and p70s6K proteins, thereby reducing the proliferation of SKOV3 cells. At the same time, the inhibitory effect of Zed on ovarian cancer transplantation and the side effects on nude mice need to be further studied, which will provide the basis for the clinical application of Zed.

In summary, this study showed that Zed can inhibit the proliferation of human ovarian cancer cell line SKOV3 and arrest SKOV3 cells at G1 phase,

and it may regulate the proliferation of SKOV3 cells through inhibiting mTOR/p70s6K signalling pathway.

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Conflict of interests

The authors declare no conflict of interests.

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