Experimental study of miR-25 targeting PTEN/PI3K/AKT signalling pathway to inhibit apoptosis of ovarian cancer SKOV3 cells.

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Abstract

This study aims to investigate whether miR-25 acts on regulating the expression of PTEN and affecting the proliferation and apoptosis of ovarian cancer cell line SKOV3. The expression of miR-25 and PTEN in the test group and the control group were compared. The targeting relationship between miR-25 and PTEN was identified by the experiment of dual-luciferase reporter gene. SKOV3 cells were cultured in vitro and were divided into five groups. Flow cytometer was used to detect the cell apoptosis and cycles and measure the expressions of miR-25 and PTEN. Western blot was used to measure the expression of proteins. The expression of miR-25 was increased and the expression of PTEN was decreased in the ovarian tumour tissues, compared to normal ovarian tissues. The expression of PTEN could be targeting inhibited by miR-25. The expression of p-AKT could be significantly inhibited by the down-regulation on the expression of miR-25 and (or) up-regulation on the expression of PTEN, and the phosphorylation of FoxO3a was significantly decreased, and the expression of p27Kip1 was increased. Moreover, cell apoptosis was significantly increased, and cell cycle was blocked at the stage G₀/G₁. Through targeting inhibition of PTEN, the abnormally over-expressed miR-25 can up-regulate the activity of PI3K/AKT signal pathway, increase the phosphorylation activity of FoxO3a and down-regulate the expression of p27Kip1, and consequently to antagonize the cell apoptosis of ovarian cancer cell SKOV3 and promote the progress of cell cycles. These may play roles in the onset of ovarian cancer.

Keywords: miR-25, PTEN, PI3K/AKT, FoxO3a, p27Kip1, SKOV3, Ovarian cancer.

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Introduction

Ovarian Cancer (OC) is one of the common malignant tumours occurred in female genital organs, and its incidence rate is only second to cervical cancer and uterine body carcinoma, ranking the third among malignant tumours of female genital system [1]. Epithelial Ovarian Cancer (EOC) is one of the most common cancers in all ovarian cancers, approximately accounting for 80%–90% of all ovarian cancer pathological types [2]. Phosphatidyl inositol 3-kinase/protein kinase B (PI3K/AKT) is an important signal transduction pathway that participates in the regulation of cell survival, which could antagonize cell apoptosis, promote cell proliferation and survival, etc. Its abnormal expression and excessive activation are correlated with tumorigenesis, tumor progression and drug resistance, etc. [3]. Phosphatase and tensin homology deleted on chromosome ten (PTEN), firstly discovered in 1997, is the only cancer suppressor gene both has the lipoprotein lipase and phosphatase activities synchronously to date, and it can play regulation roles on cell proliferation, apoptosis, migration and differentiation and other various biological processes via the negative regulation on the PI3K/AKT signal pathway, which can play roles on in promoting the cell proliferation and cell survival [4]. Decreased expression of PTEN or function defect and excessive activation of PI3K/AKT signal pathway are correlated with multiple tumors, such as, lung cancer [5], Esophageal cancer [6], Breast cancer [7], Colorectal cancer and so on [8]. Studies indicated that the expression of PTEN in tumour tissues was significantly decreased in patients with the ovarian cancer, suggesting that PTEN acts as the cancer suppressor gene in the onset of ovarian cancer [9]. Being a single-stranded non-coding micro molecular RNA that is endogenously expressed by organisms in the length of approximately 22–25 nucleotides, microRNA inhibits the translation of mRNA or degrades mRNA via complete or incomplete complementary pairing with 3'-untranslated region (3'-UTR) of the target gene mRNA, so that the expression of genes could be negatively regulated at the level of post transcription [10]. A number of studies have reported that
[11-13] the expression of miR-25 in tumour tissues or ovarian cancer cell lines was significantly increased in patients with the ovarian cancer, indicating that the increased expression of miR-25 may act as the carcinogenic gene in the onset of the ovarian cancer. Bioinformatics analysis shows that there are complementary pairing binding sites between miR-25 and 3’-UTR of PTEN mRNA. This study aims to investigate whether miR-25 plays roles in regulating the expression of PTEN and affecting the proliferation and apoptosis of cell line SKOV3 of epithelial ovarian cancer.

**Materials and Methods**

**Main reagents and materials**

Human cell line SKOV3 of epithelial ovarian cancer was purchased from the cell bank of Chinese Academy of Sciences; RPMI 1640 medium, FBS and mycillin were purchased from Gibco, U.S.A.; Liposome transfection reagent Lipofectamine 2000 was purchased from Invitrogen, U.S.A.; ReverTra Ace qPCR RT Kit and SYBR dye were purchased from Toyobo, Japan; Mouse anti-PTEN, rabbit anti-p-PTEN, rabbit anti-p-AKT were purchased from CST, U.S.A.; Mouse anti- p-AKT was purchased from Santa cruz, U.S.A.; Annexin V/PI apoptosis detection reagents were purchased from Beyotime, China; Annexin V/PI apoptosis detection reagents were purchased from Yeasen, Shanghai, China; Dual-Luciferase® Reporter Assay System and pGL3-promoter plasmid were purchased from Promega, U.S.A.

**Clinical data**

Sixty-eight patients with the epithelial ovarian cancer were enrolled into this study, who were initially diagnosed and received treatment in the hospital from January 2015 to March 2016, and the tumor samples removed during the surgery were preserved. The patients were aged from 29 to 69 y old with the mean age of 53.9 y old. All the cases were confirmed through the pathological examination, including 45 patients with serous cystadenocarcinoma and 23 patients with mucinous cystadenocarcinoma. The pathological staging conformed to the criteria for ovarian cancer specified by the International Federation of Gynecology and Obstetrics (FIGO) [14]: 8 patients were at the stage I, 14 patients were at the stage II, 21 patients were at the stage III, 15 patients were at the stage IV. Thirty cases of normal ovarian tissues incised during the surgery for patients with uterine fibroid were used as the control. All these patients were aged from 1 to 70 y old with the mean age of 53.9 y old. All patients provided a signed informed consent, and the protocols were approved by the Ethics Committee of the hospital.

**Cell culture**

SKOV3 cells were inoculated on RPMI 1640 medium containing 10% FBS and 1% mycillin, and maintained in a 5% CO₂ incubator at 37°C, and the medium was replaced every 2~3 d. Cells at logarithmic phase with good state were selected for experiments.

**Detection of gene expression by qRT-PCR**

In the reverse transcription reaction system with the total volume of 10 μL, including 1 μg total RNA, 2 μL RT Buffer (5X), 0.5 μL oligo dT+Random primer Mix, 0.5 μL RT
Enzyme Mix and 0.5 μL RNase inhibitor. ddH2O was complemented to the volume of 10 μL, the reaction conditions was 37°C for 15 min, and then, 98°C for 5 min. The obtained cDNA was preserved in the refrigerator at -20°C. With cDNA as the template, PCR amplification reaction was carried out with the action of TaqDNA polymerase. The primer sequences used were as follows:

- miR-25PF: 5'-CGGCGGCATTGCACTTGGTCTC-3',
- miR-25PR: 5'-GTGCAGGGTCCGAGGT-3';
- U6PF: 5'-ATTGGAACGATACAGAGAAGATT-3',
- U6PR: 5'-GGAACGCTTCACGAATTTG-3';
- PTENPF: 5'-CTGGTCTGCCAGCTAAAGGT-3',
- PTENPR: 5'-TCACCACACACAGGTAAACGG-3';
- p27Kip1PF: 5'-CTGGTCTGCCAGCTAAAGGT-3',
- p27Kip1PR: 5'-TCACCACACACACAGGTAAACGG-3';
- β-actinPF: 5'-GAACCCTAAGGCCAAC-3',
- β-actinPR: 5'-TGTCACGCACGATTTCC-3'.

In the PCR reaction system with the total volume of 10 μL, 5.0 μL of 2X SYBR Green Mixture, 0.5 μL of 2.5 μm/L forward and reverse primers separately, 1.0 μL of cDNA and 3.0 μL of ddH2O were added. Conditions for PCR: 95°C for 5 min, 95°C for 15 s, 60°C for 60 s. Forty cycles were amplified by the Bio-Rad CFX96 fluorescence quantitative PCR instrument, and fluorescent data were collected and analysed.

Western blot
NP40 filtered cells were used and the supernatant was collected to measure the concentration of proteins. 6 μg of supernatant was sampled and separated by 10% SDS-PAGE for 2.5 h; the product was transferred into the PVDF film for 1.5 h, and blocked by 5% skim milk powde for 60 min at room temperature. The primary antibodies were inoculated at 4°C overnight (The dilution ratio of PTEN, p-AKT, p-FoxO3a, p27Kip1 and β-actin was 1: 200, 1: 100, 1: 100, and 1: 200, 1: 1000, respectively), and then rinsed by PBST for three times. The HRP labeled secondary antibodies were incubated for 60 min at room temperature, and rinsed by PBST for three times, and then incubated at room temperature for 2 min after ECL chemiluminescence liquid was added. Subsequently, the above solution was exposed, developed and fixed in a dark room, and the data obtained were analysed following scanning.

Detection on cell apoptosis by the flow cytometer
Cells were centrifuged and collected, and then rinsed by PBS twice. Next, 100 μL of Binding Buffer was added, drawn and mixed, and then 5 μL of Annexin V-FITC and 5 μL of PI were added and reacted for 10 min in dark. Subsequently, 400 μL of Binding Buffer was added and the cells were re-suspended. Beckman FC 500MCL flow cytometer was used for detection.

Detection of cell cycle by the flow cytometer
Cells were digested by pancreatin, and rinsed with PBS twice, and then, the cells were fixed by 75% alcohol at 4°C overnight, and then rinsed with PBS for twice. Subsequently, cells were stained by the dye solution containing 20 μg/ml of RNase A, 0.1% Triton X-100 and 100 μg/ml PI at 4°C in dark for 20 min, and then the cells were tested by the flow cytometer immediately.

**Figure 1.** Disordered expression of miR-25 and PTEN in EOC tissues. (A) Detection on the expression of PTEN mRNA in normal ovarian tissue and ovarian cancer tissues with different clinical stages by qRT-PCR; (B) Detection on the expression of miR-25 in normal ovarian tissue and ovarian cancer tissues with different clinical stages by qRT-PCR; (C) Detection on the expression of PTEN proteins in normal ovarian tissue and ovarian cancer tissues with different clinical stages by Western blot.

**Figure 2.** Targeting inhibition on the expression of PTEN by miR-25. (A) T-Schematic diagram of the binding site between miR-25 and PTEN 3'-UTR in the prediction of microRNA.org target gene mRNA; (B) Detection of relative luciferase activity in HEK293 cells transfected with mimic NC and miR-25 mimic by dual luciferase reporter gene experiments; (C) Detection on the expression of miR-25 and PTEN in SKOV3 cells transfected with mimic NC and miR-25 mimic by qRT-PCR; (D) Detection on the expression of PTEN proteins in SKOV3 cells transfected with mimic NC and miR-25 mimic by Western blot assay.

**Statistical analysis**
Statistical analysis of data was performed by SPSS 18.0. Measurement data were expressed by mean ± standard deviation. The student t test was used in the comparison of inter-group measurement data, and P<0.05 indicated that the difference was statistically significant.
Figure 3. Down-regulation on the expression of miR-25 can inhibit the proliferation of SKOV3 cells and promote their apoptosis. (A) qRT-PCR was used to detect the expression of miR-25 and PTEN in the SKOV3 cells of different transfection groups, in order to detect the interference efficiency of miR-25 and the over expression efficiency of PTEN, and to further verify the regulatory relationship between miR-25 and PTEN; (B) Western blot was used to detect the expression of PTEN gene in SKOV3 cells of different transfection groups, the downstream effects of p27Kip1 and FoxO3a, and the activity of PI3K/AKT pathway; (C) Effect of down regulation of miR-25 or over expression of PTEN on apoptosis of SKOV3 cells by flow cytometry; (D) Effect of down-regulation of miR-25 or over expression of PTEN on cell cycle of SKOV3 cells by flow cytometry. (Notes: * represents the comparison between miR-25 inhibitor and inhibitor NC, P<0.05; # represents the comparison between pIRES2-PTEN and pIRES2-Scramble, P<0.05; Δ represents the comparison between miR-25 inhibitor+pIRES2-PTEN and inhibitor NC, P<0.05; & represents the comparison between miR-25 inhibitor+pIRES2-PTEN and pIRES2-Scramble, P<0.05.)
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Results

Disordered expression of miR-25 and PTEN in EOC tissues

The results of qRT-PCR showed that the expression level of PTEN mRNA in tumour tissues in patients with EOC was significantly lower than that of normal ovarian tissues (Figure 1A), while the expression level of miR-25 was obviously higher than the latter (Figure 1B), and all these were correlated with the pathological staging of FIGO. The results of Western blot assay indicated that compared to normal ovarian tissues, the expression of PTEN proteins was significantly decreased in tumour tissues for patients with EOC (Figure 1C), and the more advanced pathological staging of FIGO was, the more decreased expression level was. The results suggested that the abnormal overexpression of miR-25 may decrease the expression of PTEN and promote the occurrence of EOC.

Targeted inhibition on the expression of PTEN by miR-25

The target gene prediction results from microRNA, org online showed that there were targeted complementary pairing binding sites between miR-25 and 3’-UTR of PTEN mRNA (Figure 2A). The results of dual-luciferase reporter gene experiments indicated that the transfection of miR-25mimic significantly decreased the relative activity of the luciferase in HEK293 cells (Figure 2B) and the expression of PTEN in SKOV3 cells (Figures 2C and 2D). However, the transfection of mimic NC had no significant inhibition on the relative activity of luciferase in HEK293 cells and the expression of PTEN in SKOV3 cells, suggesting that miR-25 could targeting inhibit the expression of PTEN.

Down-regulation on the expression of miR-25 can inhibit the proliferation of SKOV3 cells and promote their apoptosis

Down-regulation on the expression of miR-25 and (or) up-regulation on the expression of PTEN can significantly inhibit the expression of p-AKT (Figures 3A and 3B) and obviously reduce the phosphorylation of FoxO3a (Figure 3B). Moreover, the expression of p27Kip1 was significantly enhanced (Figure 3B) and the cell apoptosis (Figure 3C) was significantly increased, and cell cycles were blocked at the stage G0/G1 (Figure 3D).

Discussion

It is estimated that 225,000 patients are newly diagnosed with ovarian cancer every year throughout the world [15], and patients who die of ovarian cancer reach up to 145,000 every year [16]. There are significant differences in the regional distribution for ovarian cancer. The incidence rate in western developed countries is higher (approximately 10~12/100,000) than that in China (about 2~3/100,000). However, the incidence rate of ovarian cancer in China has been increasing year by year recently [15,17]. According to statistics [18], the mortality rate of ovarian rate reaches up to 5~7/100,000 throughout the world and ranks the first among malignant tumours of female reproductive system, which seriously endangers the life and health of females. The development of ovarian cancer is relatively hidden, and no significantly clinical symptoms were found at the early stage. However, this disease is progressed quickly, and approximately 60%~70% of patients have been in the middle and advanced stage when they feel discomfort and seek for medical treatment [19]. To date, no satisfactory clinical effects are obtained in the dominated surgical excision treatment, supplemented by chemotherapy and radiotherapy measures, and the qualities of lives of patients are not significantly improved. The 5 y survival rate is still lower being about 20~30% [20]. In general, the recurrence rate of ovarian cancer is higher for its characteristics of easy to metastasis and acquiring drug resistance, and approximately 50%~70% of patients will suffer from re-occurrence within 18 months after the first-line treatment [21].

Nearly all the development processes of tumours involve the changes of multiple factors and pathways of overexpression of proto oncogenes, viability and function defects of cancer suppressor genes, and PI3K/AKT signal transduction pathway extensively participates in the regulation of cell proliferation, cycle, apoptosis, differentiation and other biological effects, and is closely correlated with the occurrence, progression, treatment and outcome of malignant tumours [22]. PI3K, an important member in phospholipids kinase family, is formed by the third inositol phosphorylation of the phosphoinositide ring on the cell membrane and consists of catalytic subunit P110 and regulatory subunit P85. Under the stimulation of extracellular signals, PI3K is recruited into the site adjacent to the plasma membrane to catalyse the phosphorylation of its substrate phosphatidylinositol 4, 5-trisphosphate (PIP2) to generate phosphatidylinositol 3, 4, 5-trisphosphate (PIP3) [23]. AKT, also named protein kinase B (PKB), is a specific protein kinase to serine/threonine, and is one of downstream target molecules of PI3K. AKT under the rest state is usually distributed in the cytoplasm, however, under the action of the second messenger PIP3, the lipid binding site of AKT (i.e., PH binding site) can specifically combine with PIP3, which induces the conformational changes in the tertiary structure of AKT, as a result, it transfers from the cytoplasm to the cell membrane, and the two main phosphorylated residues of AKT are exposed. Under the catalytic action of PI3K-dependent kinase 1 (PDK1) and PI3K-dependent kinase 1 (PDK2), Thr308 and Ser473 sites of AKT are phosphorylated, and the phosphorylated and activated AKT can continuously deliver the information of promoting the cell growth and proliferation and inhibiting the cell apoptosis to downstream [24]. FoxO3a, an important member in Fork Head Transcription Factor (FKHR) family, can up regulate the expression of pro-apoptotic genes, for example, specific Cyclin-dependent Kinase Inhibitor (CKI) p27Kip1 [25], Bim [26], PUMA [27], or down-regulate the expression of antiapoptotic gene FLIP [28], and play roles on promoting cell apoptosis. Cell cycle regulatory protein p27Kip1 can closely combine with CDK2 and CDK4, inhibit their activities and block cells from entering...
into DNA synthetic stage S, thus blocking cell cycles at the stage G_{0}/G_{1} and negatively regulating the proliferation of cells [29]. Phosphorylated and activated AKT in PI3K/AKT signal pathway can further phosphorylate FoxO3a to transfer FoxO3a from the nucleus to cytoplasm and reduce its transcriptional regulation on the nuclear genes [30], while the dephosphorylated FoxO3a immediately enters into the nucleus from the cytoplasm to trigger the transcription and translation of target genes and accelerate the cell apoptosis [31]. As a cancer suppressor gene, PTEN can inhibit the activity of phosphatase through dephosphorylation of PIP3 [32], or directly removing the phosphate group on serine/threonine residues of AKT, so as to antagonize the regulation of PI3K/AKT signal pathway on cell proliferation, cycle and apoptosis [33]. Studies indicated [9] that the expression of PTEN in tumour tissues was significantly decreased for patients with the ovarian cancer, suggesting that PTEN can act as a cancer suppressor gene in the onset of ovarian cancer. A number of studies reported that [11-13] the expression of miR-25 in tumour tissues or ovarian carcinoma cells were significantly increased in patients with the ovarian cancer, indicating that the increased expression of miR-25 may act as the carcinogenic gene in the onset of the ovarian cancer. Bioinformatics analysis shows that there are complementary pairing binding sites between miR-26a and 3'-UTR of PTEN mRNA. This study aims to investigate whether miR-25 plays roles on regulating the expression of PTEN and affecting the proliferation and apoptosis of cell lines SKOV3 of the epithelial ovarian cancer.

The results of this study indicated that compared to normal ovarian tissues, the expression of PTEN in epithelial ovarian cancer tissues was significantly decreased, and the expression of miR-25 was significantly increased. With the increasing of pathological staging of FIGO, the expression level of PTEN was gradually decreased, while the expression of miR-25 was increased, suggesting that the high expression of miR-25 may play roles in down regulating the expression of PTEN and promoting the onset of EOC. The studies performed by Yang et al. [34] showed that the expression level of PTEN was significantly decreased in tumour tissues for patients with ovarian cancer. The studies performed by Schondorf et al. [35] showed that the expression of PTEN was abnormally reduced in tumour tissues for patients with the ovarian cancer, furthermore, the more decreased the expression level was, the worse the prognosis was and the patients' conditions were more prone to progressed or aggravated. The studies performed by Chen et al. [36] indicated that the detection rate of the 5th exon gene mutation was higher on the PTEN gene for patients with EOC, so that the expression level of PTEN mRNA in tumour tissues was significantly lower than that of normal ovarian tissues or ovarian cyst tissues. The present study found that the expression level of PTEN was abnormally decreased in cancer tissues of patients with ovarian cancer, which is basically consistent with the results of the above studies. This indicates that the reduction of PTEN expression was involved in the onset of ovarian cancer. The studies performed by Wang et al. [13] demonstrated that the expression level of miR-25 in tumour tissues of patients with EOC was significantly higher than that of para-carcinoma tissues, and its expression level was significantly correlated with the pathological staging and pathological differentiation; The six-year survival rate of patients with higher expression of miR-25 was only 15.96%, significantly lower than patients with lower expression of miR-25 being 45.89%. The studies performed by Feng et al. [11] showed that the expression level of miR-25 was abnormally increased in tumour tissues and cancer cell lines of ovarian tissues. The studies performed by Zhang et al. [12] also found that the expression of miR-25 in tumour tissues for patients with ovarian cancer was higher than that of normal ovarian tissues, and the expression level of miR-25 in ovarian cancer cell lines SKOV3, OVCAR3, OVCAR5 and A2780 was obviously higher than that of Ovarian Surface Epithelial cells (OSE). The results of this study showed that the expression level of miR-25 in cancer tissues for patients with EOC was obviously higher than that of normal ovarian tissues, which was consistent with the results of Feng et al. [11] and Zhang et al. [12]. The results of dual-luciferase reporter gene experiments showed that the transfection of miR-25 mimic could significantly reduce the relative activity of luciferase in HEK293 cells and the expression of PTEN in SKOV3 cells, proving that there were targeted regulatory relations between miR-25 and PTEN. Further detection showed that the down-regulation on the expression of miR-25 and (or) up-regulation on the expression of PTEN can significantly inhibit the expression of p-AKT, and significantly decrease the phosphorylation of FoxO3a and increase the expression of p27Kip1. Moreover, the cell apoptosis was significantly increased, and the cell cycle was blocked at the stage G_{0}/G_{1}. The studies performed by Zhang et al. [12] indicated that the down-regulation on the expression of miR-25 can significantly induce the apoptosis of ovarian cells SKOV3 and OVCAR3, and the overexpression of miR-25 can obviously promote the proliferation of SKOV3 and OVCAR3. Both the stimulated proliferation and antiapoptotic effects of miR-25 on ovarian cancer cells would be realized through the targeted inhibition on the expression of cancer suppressor gene BIM. The studies performed by Feng et al. [11], indicated that miR-25 can promote the proliferation, migration and invasion and other biological functions of ovarian cancer cells through the targeting inhibition on the expression of Large Tumour Suppressor 2 (LATS2), so as to act as the carcinogenic gene in the onset of ovarian cancer. This study observed that miR-25 could inhibit the apoptosis of ovarian cancer cells SKOV3 and promote cell cycles, suggesting that the increased expression of miR-25 was correlated with the development of ovarian cancer and malignant biological characteristics of cells, which was basically consistent with the results of Feng et al. and Zhang et al. [12]. The study results of Feng et al. [37] indicated that the decreased expression of the pro-apoptosis gene FoxO3a was significantly correlated with the poor prognosis of patients with ovarian cancer. The study results of Lu et al. [38] indicated that the expression of FoxO3a was significantly decreased in tumour tissues for patients with ovarian cancer. Lu et al. also [39] revealed that the significantly increased phosphorylation of FoxO3a in ovarian cancer tissues was positively correlated
Conclusion

The expression of miR-25 was significantly increased and the expression of PTEN was significantly decreased in ovarian tumour tissues. Through the targeting inhibition on PTEN, the abnormally increased expression of miR-25 could up-regulate the activity of PI3K/AKT signal pathway, increase the phosphorylation activity of FoxO3a and down-regulate the expression of p27Kip1, and consequently to antagonize the cell apoptosis of ovarian cancer cell SKOV3 and promote the progress of cell cycles. These may play roles in the onset of ovarian cancer.

Acknowledgement

None

Declaration of Interest

The authors declare no conflict of interest.

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