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ORIGINAL ARTICLE

Expression of PKG2 in ovarian cancer and its effect on epidermal growth factor receptor

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Summary

Purpose: *This research tried to explore the expression level of II cGMP-dependent protein kinase (PKG2) in human ovarian tissue and to clarify the molecular mechanism of EGFR regulation and its clinical significance.*

Methods: *The expression levels of PKG2 and EGFR in 10 normal ovarian tissues, 14 benign ovarian tumor tissues and 39 epithelial ovarian cancer tissues preserved in the archives of the Affiliated Hospital of Xuzhou Medical University from 2016 to 2018 were detected by real-time fluorescence quantitative (RT-PCR), and the correlation between the expressions of the two genes was analyzed. The expressions of in vitro cultured ovarian cancer cell lines SKOV3, PKG2 and EGFR were detected by RT-PCR and western blot, and the over-expressed PKG2 plasmid and PKG2 small interfering RNA (siRNA) were transfected into the cells, and the protein and phosphorylation of Akt and ERK in EGFR and its downstream signaling pathway were detected by western blot.*

Results: *Compared with normal ovarian tissue, the mRNA and protein expression levels of PKG2 in ovarian cancer tissue and SKOV3 cell line were significantly reduced ($p<0.05$). However, the mRNA and protein expression levels of EGFR in ovarian cancer tissue and SKOV3 cell line were both high ($p<0.05$). In addition, after transient transfection of PKG2, the expression changes of PKG2 significantly affected the expression of EGFR, and PKG2 over-expression could significantly inhibit the phosphorylation of Akt and ERK in EGFR and its downstream signaling pathways, thereby affecting cell proliferation.*

Conclusion: *PKG2 may play a role in inhibiting EGFR expression in ovarian cancer, but the specific mechanism of its effect on tumor development still needs to be further explored.*

Key words: *clinical significance, EGFR, ovarian cancer, PKG2, regulation*

Introduction

According to the 2018 Global Cancer Statistics Report, cancer is still one of the major diseases with the highest incidence and mortality in the world. However, as a common malignant tumor in females, ovarian cancer has the highest mortality rate [1,2]. At present, the cause of ovarian malignant tumors is still not very clear, with the main inducing factors being genetic and endocrine. Because the early stage of ovarian cancer is mostly asymptomatic, there are nearly 70% of patients being diagnosed as advanced stage [3-5].

In recent years, the key methods of treating ovarian malignant tumors are still chemotherapy and surgery. Although routine therapy can improve the outcome of some patients, more than half of the patients are diagnosed with locally advanced stage, therefore, for these patients, chemotherapy and surgery treatment cannot effectively improve patients' life. The key for a satisfactory result for these patients is early diagnosis and early treatment.

With the in-depth research of human genomics and proteomics, a large number of abnormal genes

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and protein expression profiles that are involved in tumor genesis and development have been gradually established and improved [6,7]. Nevertheless, the research on markers of early diagnosis is still urgent, so the early screening and detection of ovarian cancer still needs to be further studied. Previous researches have found that EGFR is highly abnormal in ovarian cancer, which can be used as a new biomarker for early diagnosis of this disease [8,9], but its specific regulatory mechanism is still unclear. cGMP-dependent protein kinase (PKG) is a serine/threonine protein kinase, which is mainly divided into PKG1 and PKG2, affecting the activities of cells by phosphorylation of proteins. At present, research has shown that PKG2 can inhibit the activation of EGFR itself and its downstream signaling pathways by phosphorylation of EGFR in gastric cancer, thereby affecting the proliferation, metastasis and invasion of tumor cells and other vital activities [10,11]. However, it is not clear whether EGFR is regulated by PKG2 in cells and tissues in ovarian cancer. Therefore, the purpose of this research is to investigate the expression of PKG2 in ovarian cancer and its regulation mechanism of EGFR.

Methods

Materials

Human ovarian cancer cell line SKOV3, purchased from ATCC; RPMI 1640 culture medium (Gibco, USA); fetal bovine serum (FBS) (Gibco, USA); SYBR Premix Ex Taq (TaKaRa, Japan); RNeasy mini kit (Qiagen); PrimeScript RT reagent Kit (TaKaRa, cat no. RR047A); anti-PKG2 antibody (ThermoFisher, cat no. PV3937); anti-EGFR antibody (CST, cat no. 4267s); anti-pEGFR antibody (CST, cat no. 3777T); anti-Akt antibody (CST, cat no. 4691s); anti-pAkt antibody (CST, cat no. 4060T); anti-Erk antibody (CST, cat no. 4370T); anti-pErk antibody (CST, cat no. 4370T); β-Actin antibody (CST, cat no.3700s); Goat anti-mouse IgG/horseradish peroxide recorded secondary antibodies (CST, cat no.7076s); Cell Counting Kit-8/CCK8 kit (Tongren, Japan, C0037).

Table 1. Design of primers for PKG2, EGFR and GAPDH gene amplification

Name	*Sequence of primers (5'-3')*	*Length of amplification*
PKG2-F:	TTGATTCGGAGGCTTTGCAG	
PKG2-R:	TCCCTTGAGCTCTCTTTGCA	160bp
EGFR-F:	GCGCTACCTTGTCATTCAGG	
EGFR-R:	TATCAATGCAAGCCACGGTG	227bp
GAPDH-F:	GAGACAACGGATTTGGTCG	
GAPDH-R:	TTAGATTTTGGAGGGATCTC	238bp

F for Forward, R for Reverse

Source of sample tissues

Pathological tissue samples from patients admitted to the Affiliated Hospital of Xuzhou Medical University with ovarian tumor from 2016 to 2018 and retained after surgery were collected. Paracancer tissue in patients with ovarian cancer after resection were defined as normal tissue of human ovary. Benign ovarian tumor tissue and epithelial ovarian cancer (malignant tumor) tissue were removed in small pieces of 0.3-0.5 cm after surgery and put into RNA protective fluid (Qiagen, cat #76106, Germany), and kept in liquid nitrogen of -196°C for follow-up experiments. The clinical sample organizations of the patients used in this study were approved by the Clinical Ethics Committee of the Affiliated Hospital of Xuzhou Medical University and all patients had signed the informed consent.

Cell culture

Human ovarian cancer cell line SKOV3 was routinely cultured in RPMI 1640 complete culture medium (containing 10% FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2 cell incubator at 37°C. When the cell culture reached 80-90% confluence of the bottom area of the bottle, trypsin-EDTA cells were digested, centrifuged and subcultured, and the cells at logarithmic growth phase were selected for follow-up experiments.

Expression levels of PKG2 and EGFR mRNA in ovarian cancer tissues and cell lines by quantitative RT-PCR detection

Tissue RNA was extracted from 10 cases of normal ovarian tissues, 14 cases of benign ovarian tumor tissues and 39 cases of epithelial ovarian cancer tissues preserved in liquid nitrogen screened from ovarian cancer tissue samples. Meanwhile, human ovarian cancer cell line SKOV3 in logarithmic growth phase was collected, and total RNA of cells was extracted by RNeasy mini kit from Qiagen Co. The concentration and purity of RNA were measured by Nucleic Acid Detector, and the best RNA purity was between A260/A280=1.8~2.0. The TaKaRa reverse transcription kit was used to convert RNA reverse transcription into cDNA for quantitative RT-PCR, with GAPDH as the internal reference. Each experiment was repeated three times. The design for primer is shown in Table 1.

Western blot assay

Total protein was extracted from RIPA lysate containing 1% protease inhibitor, and protein expression levels of PKG2 and EGFR in human ovarian cancer cells were detected by polyacrylamide gel electrophoresis, with β-actin as the internal reference. Gel electrophoresis was performed at a constant pressure of 90V for about 1.5-2h. After the completion of electrophoresis, semi-dry method was used to transfer the membrane. About 1h later after transferring the membrane, 5% skim milk powder sealing solution (5g/100mL) was used for 1h at room temperature. The dilution ratio of primary anti-PKG2 was 1 to 1000, the dilution ratio of primary anti-EGFR was 1 to 1000, and the dilution ratio of β-actin was 1 to 2000. The dilution ratio of the secondary antibody

- goat anti-rat - was 1 to 3000. Finally, EZ ECL enhanced ChemiScope (Good No.: D3030L1260) was used for developing and for imaging the detection in CLINX ChemiScope imaging system (Type: ChemiScope 3600 Mini).

Cell transfection

SKOV-3 cells were placed into 24-well plates with 2×10^5 cells per well. After the cells were cultured for 24h, covering the volume of the board about 70-80%, the upper culture RMPI 1640 medium was removed. The transfection complex was configured using lipofectamine 3000 as the transfection reagent (according to the operating instructions), added to cells with a volume of 0.5 mL/drop into each well, and 6h later, new complete culture RPMI 1640 was changed. Forty-eight h later, cells were collected and their total RNA and protein were extracted for follow-up experiments.

Cell proliferation detected by CCK8 assay

SKOV3 cells were put into a 96-well plate board with 2.5×10^3 cells into each well, and PKG2 overexpressed plasmid and PKG2 siRNA were constructed. Then, lipofectamine 3000 transfection reagent was used to transfect SKOV3 cells, and 10 µl of CCK-8 solution were added to each well separately on days 0, 1, 2, 3 and 4 after transfection. After shaking and mixing, the cells were incubated at 37°C for 3-4h. The optical density (OD) value of each well was determined at the wavelength of 450 nm by micrometer, and the cell proliferation activity was proportional to the OD value. The experiment was repeated three times.

Statistics

Data in this research were plotted by GraphPad Prism5 and statistically analyzed by SPSS18.0. All experimental results were repeated three times. The experimental data were expressed as mean ± standard deviation, and the t-test was used for comparison between the two groups. P<0.05 indicated that the difference has statistically significance.

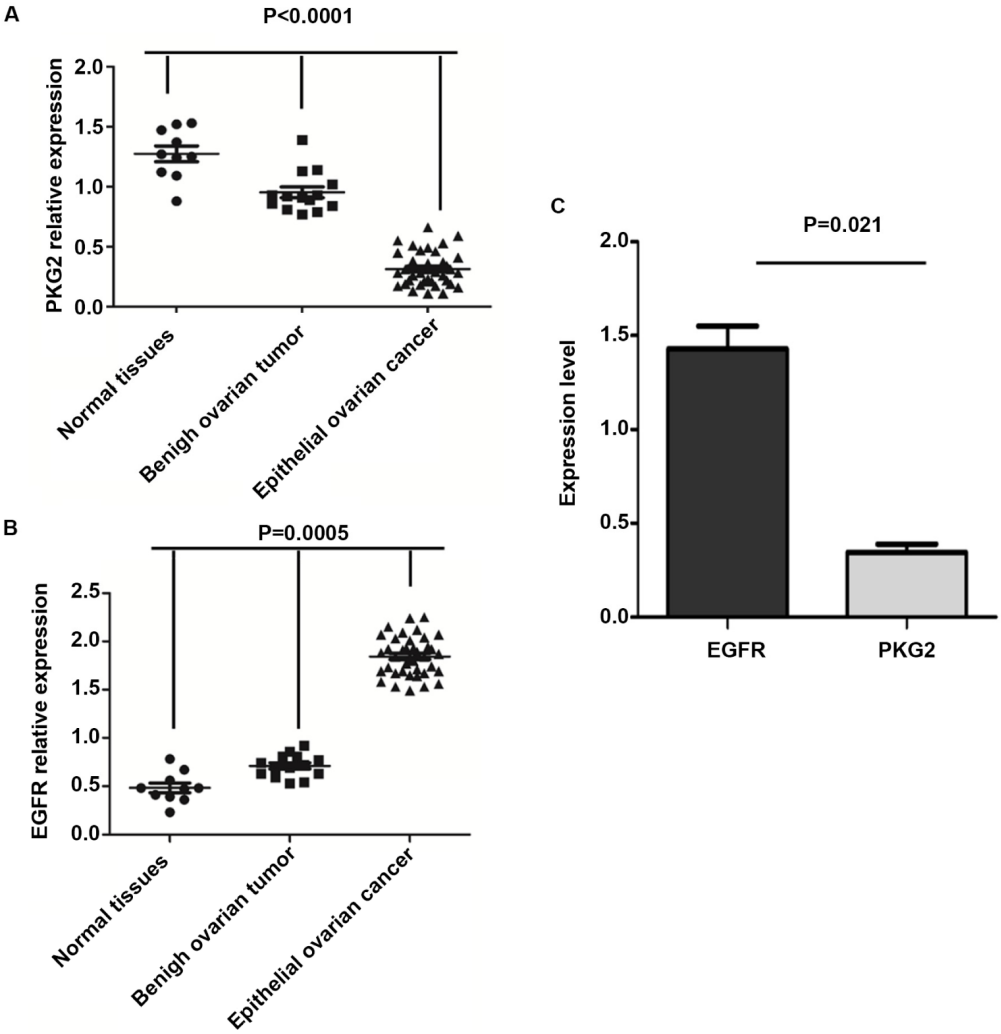

Figure 1. The expression levels of PKG2 and EGFR in normal and benign ovarian tissues and ovarian cancer cell line SKOV3 were detected by real-time fluorescence quantitative RT-PCR. **A:** PKG2 as expressed in normal ovarian tissues, benign ovarian tumor tissues and epithelial ovarian cancer tissues. **B:** EGFR expression as expressed in normal ovarian tissues, benign ovarian tumor tissues and epithelial ovarian cancer tissues; **C:** expression levels of PKG2 and EGFR in cell line SKOV3.

Results

Expression of PKG2 and EGFR in human ovarian cancer tissues and SKOV3 cell line detected by quantitative RT-PCR

According to real-time fluorescence quantitative RT-PCR, it was shown that the mRNA expression level of PKG2 in epithelial ovarian cancer tissues was significantly lower than that in normal ovarian tissues and benign ovarian tumor tissues ($p<0.0001$) . In contrast, the mRNA expression level of EGFR in epithelial ovarian cancer tissues was significantly higher than that in normal ovarian tissues and benign ovarian tumor tissues

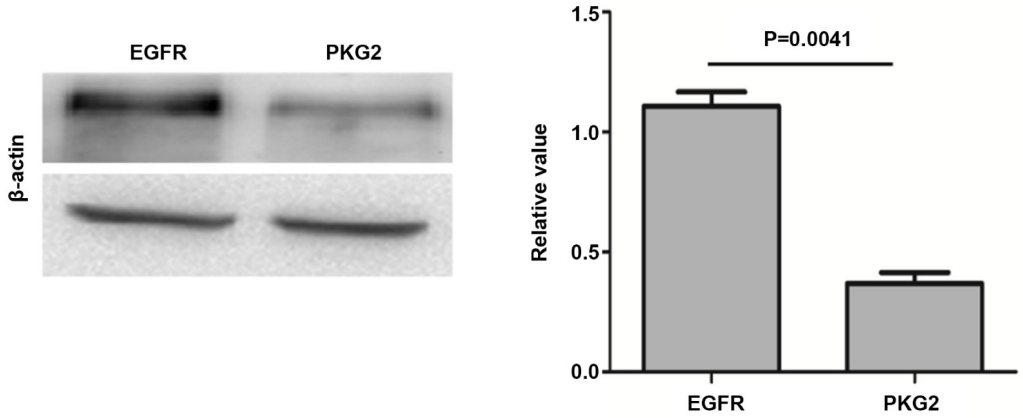

Figure 2. Protein expression levels of EGFR and PKG2 in ovarian cancer cell lines detected by Western blot.

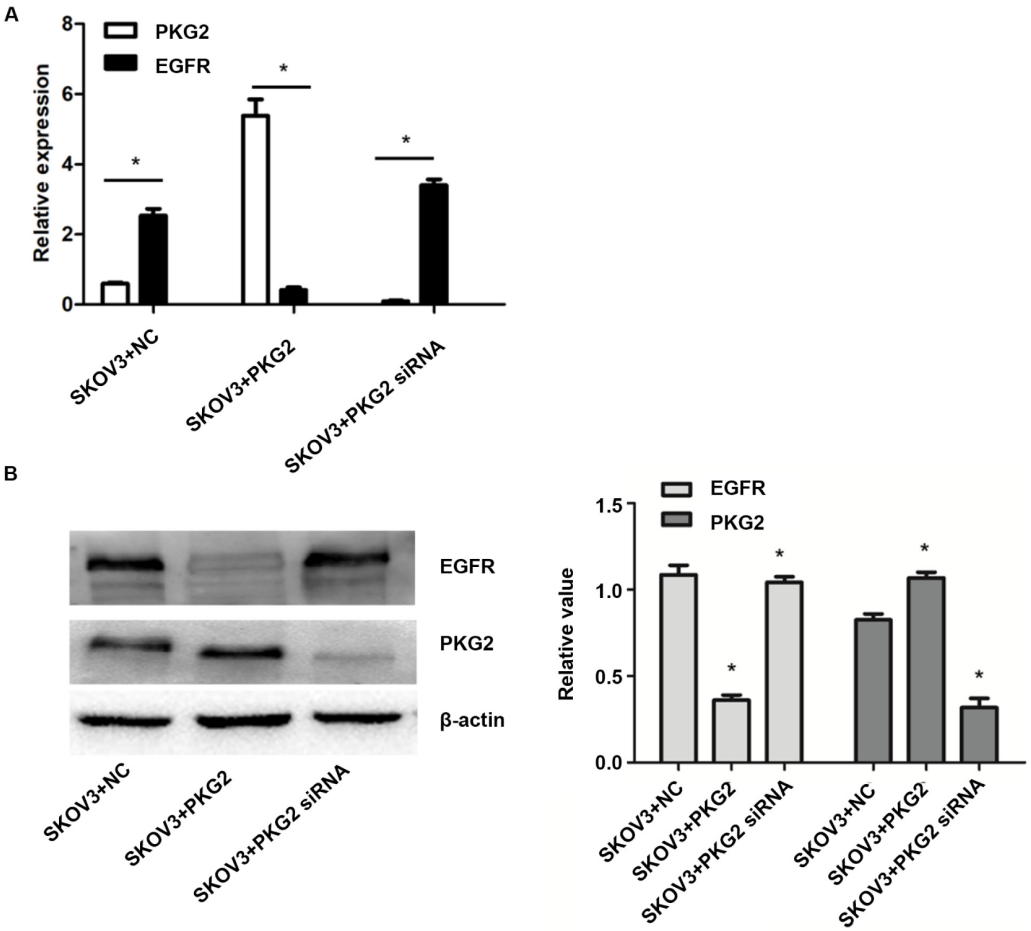

Figure 3. RT-PCR and Western blot analysis to detect the expression levels of PKG2 and EGFR in mRNA and protein, when PKG2 overexpression plasmid and PKG2 siRNA were transiently transfected to SKOV3. **A:** RT-PCR was used to detect the mRNA level of the target gene; **B:** Western blot was used to detect the expression level of the target gene protein. (*$p<0.05$).

(p=0.0005). However, although there were differences in PKG2 and EGFR expression levels between normal ovarian tissues and benign ovarian tumor tissues, the differences were not statistically significant (Figure 1A/B). Similar to ovarian cancer tissues, PKG2 expression was low in ovarian cancer cell line SKOV3, but the expression level of EGFR was significantly higher, with a statistically significant difference between the two (p=0.021) (Figure 1C).

Expression of PKG2 and EGFR in human ovarian cancer cell lines detected by Western blot

Total RNA and total protein were extracted from SKOV3 cell line of human ovarian cancer. Quantitative RT-PCR and Western blot detection revealed that the mRNA and protein expression levels of PKG2 in SKOV3 cells were significantly lower than the EGFR expression levels, with statistical differences (p<0.05) . β-actin was used as internal reference, as it is shown in Figure 2.

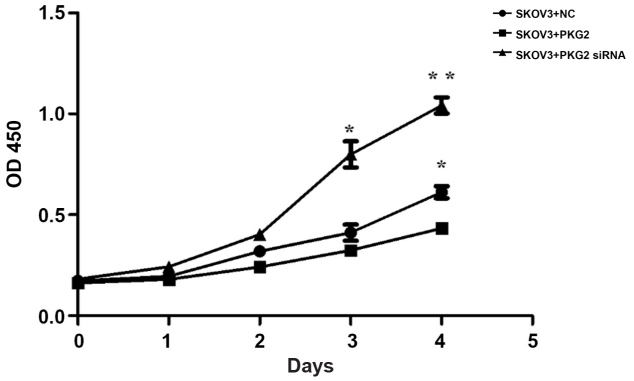

Figure 4. Cell proliferation detected by CCK8 assay (*p<0.05; **p<0.01).

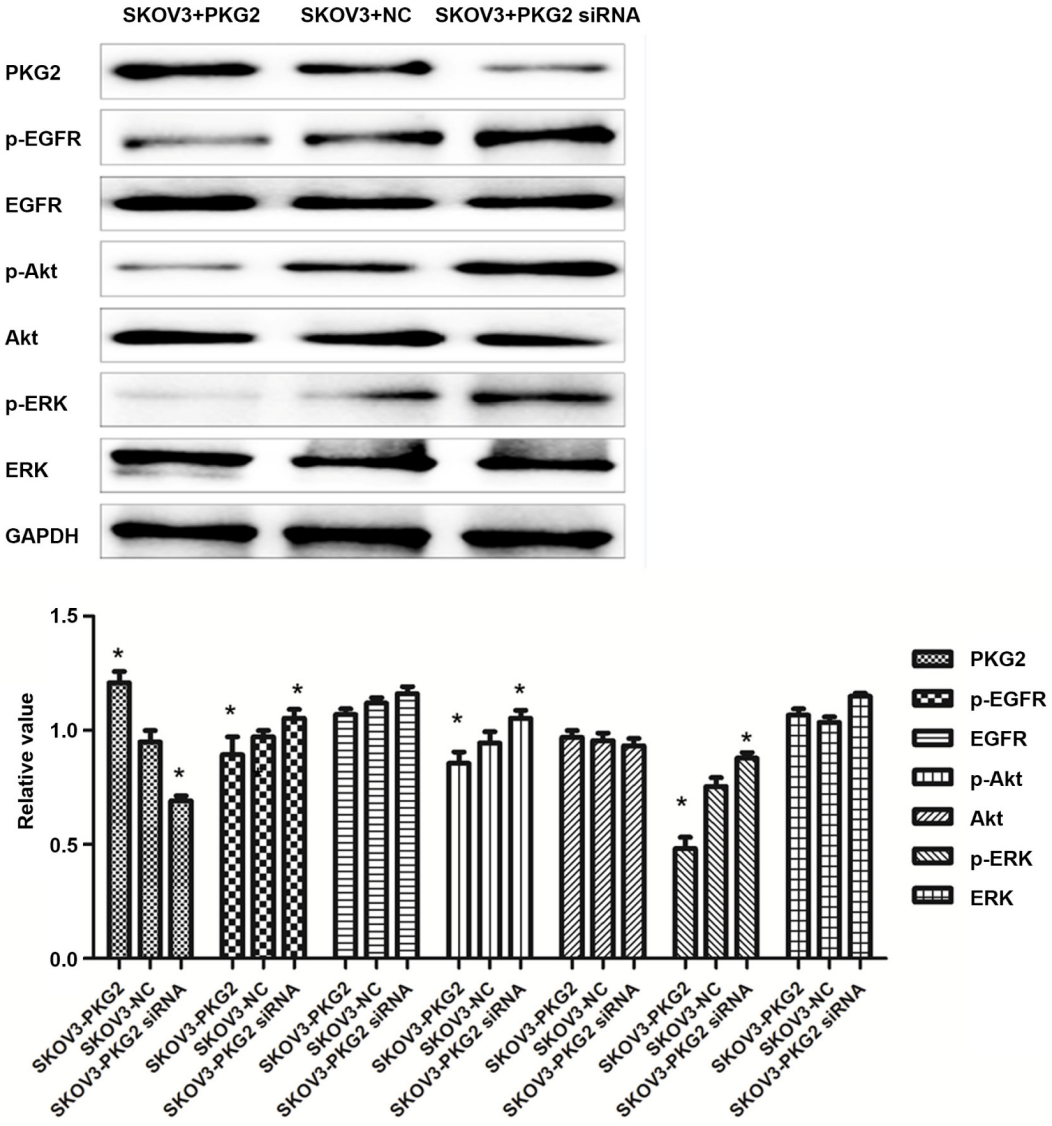

Figure 5. Phosphorylation levels of AKT and ERK in the downstream signaling pathway of EGFR detected by Western blot (*p<0.05).

EGFR expression detected by transient transfection of PKG2 overexpressed plasmid or PKG2 siRNA into SKOV3 cells

The expression changes of PKG2 and EGFR after transfection with PKG2 overexpressed plasmid or siRNA were detected by RT-PCR and Western blot. The expression level of EGFR increased significantly after PKG2 was silenced. Therefore, the expression of PKG2 inhibited the expression of EGFR in ovarian cancer cells, showing a statistically significant difference, as it is shown in Figure 3 ($p<0.05$).

Effects of abnormal PKG2 expression on the proliferation of ovarian cancer cell lines

After transient transfection of PKG2 overexpressed plasmid or PKG2 siRNA into SKOV3 cells, SKOV3 cell proliferation was detected by CCK8 cell proliferation detection kit on days 0, 1, 2, 3 and 4, respectively. The results showed that PKG2 overexpression inhibited the proliferation of ovarian cancer cells, as it is shown in Figure 4.

Effect of abnormal PKG2 expression on the downstream signaling pathway of EGFR

After transient transfection of PKG2 overexpressed plasmid or PKG2 siRNA into SKOV3 cells, Western blot was used to detect the changes of PKG2 expression in the downstream EGFR signaling pathway of AKT and ERK proteins and their phosphorylation. The results showed that PKG2 overexpression inhibited the phosphorylation of AKT and ERK in EGFR phosphorylation and its downstream signaling pathways, as it is shown in Figure 5.

Discussion

The occurrence of cancer is a multi-gene, multi-stage, multi-step process. As other malignancies, the occurrence and development of ovarian cancer is jointly involved by various genetic and epigenetic factors. Phosphorylation is a kind of epigenetics, which is widely involved in the genesis and development of tumors. EGF induces phosphorylation of EGFR and activates its downstream PI3K/Akt, MAPK/ERK signaling pathways, which are widely involved in the proliferation, invasion, metastasis and angiogenesis of tumor cells [12-15]. At present, previous research has thoroughly elucidated the functions of EGFR in non-small cell lung cancer, gastric cancer, colorectal cancer, breast cancer, etc [16-19]. In ovarian cancer, EGFR has been reported as a potential diagnostic marker of ovarian cancer, but its regulatory mechanism still needs to be further studied [20,21].

PKG2 is a serine/threonine protein kinase, which constitutes two members of the PKG protein family together with PKG1. In gastric cancer, PKG2 can inhibit the changes in protein expression caused by EGF and reverse the changes in phosphorylation levels of various proteins caused by EGF [10]. Therefore, we hypothesized that PKG2 may play a similar role in ovarian cancer.

In this research, we found that the expression level of PKG2 in epithelial ovarian cancer tissues was significantly lower than that in normal ovarian tissues and benign ovarian tumor tissues by quantitative RT-PCR and Western blot, with a statistical difference ($p<0.05$). In order to confirm the correlation between PKG2 and EGFR, we transfected ovarian cancer cell line SKOV3 by constructing PKG2 expression plasmid and PKG2 siRNA, and the results showed that the abnormal expression PKG2 would significantly change the expression of EGFR. PKG2 expression can inhibit EGFR phosphorylation levels, and further reduce the EGF and EGFR signaling pathways downstream of ERK and Akt phosphorylation level, which has a significant impact on tumor cell proliferation. In ovarian cancer cells and tissues, the low expression level of PKG2 leads to high phosphorylation levels of Akt and ERK proteins in EGFR and its downstream signaling pathways, which are widely involved in the proliferation and invasion of tumor cells.

Preliminary results in this research verify that PKG2 could inhibit EGFR protein expression in ovarian cancer and its phosphorylation level, and preliminarily proved that PKG2 has further influence on EGFR downstream signaling pathways. However, we still don't know if phosphorylation of EGFR by PKG2 has direct effect on EGFR phosphorylation sites, and which sites were changed. This issue merits further study. In conclusion, through this research, we preliminarily revealed a new mechanism by which EGFR is regulated by PKG2 expression in ovarian cancer, and provided more experimental basis for PKG2 to possibly participate in the occurrence and development of ovarian cancer as an anticancer gene.

Conflict of interests

The authors declare no conflict of interests.

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