

## Original Article

# Reversal of cisplatin resistance in ovarian cancer cells mediated by naringin-induced COX-2 expression through the NF- $\kappa$ B signaling pathway

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**Abstract:** Objective: To investigate the effect of naringin on COX-2 expression in SKOV3/DDP cell lines and the mechanisms of potential signaling pathways. Methods: The effect of naringin at different concentrations on the expression of NF- $\kappa$ B and COX-2 in SKOV3/DDP cell lines was detected by RT-PCR and Western blot, respectively; the effect of NF- $\kappa$ B on the expression of COX-2 mRNA was studied by targeted silencing and overexpression of NF- $\kappa$ B, and bidirectional adjustment of NF- $\kappa$ B expression in SKOV3/DDP cells. In SKOV3/DDP cells overexpressing NF- $\kappa$ B, naringin was added and cultured for 48 hours before the expression of COX-2 mRNA was detected. Results: RT-PCR and Western blot showed that the gene and protein expression of NF- $\kappa$ B and COX-2 in SKOV3/DDP cells gradually decreased with the increase of naringin concentration. After overexpression of NF- $\kappa$ B, the expression of COX-2 increased; on the other hand, the expression of COX-2 was down-regulated after NF- $\kappa$ B was silenced; the high expression of COX-2 induced by overexpression of NF- $\kappa$ B could be down-regulated by naringin. Conclusion: Naringin can inhibit the expression of NF- $\kappa$ B and COX-2 in SKOV3/DDP cells, and such inhibition may be related to the regulation of NF- $\kappa$ B signaling pathway, which may be one of the mechanisms involved in the reversal of cisplatin resistance in ovarian cancer cells by naringin.

**Keywords:** Ovarian cancer, naringin, COX-2, cisplatin resistant, NF- $\kappa$ B signaling pathway

## Introduction

Cisplatin resistance in ovarian cancer is one of the main reasons for the failure of its clinical treatment. Cyclooxygenase (COX) is an important rate-limiting enzyme in prostaglandins (PG) synthesis, wherein COX-2 is an important oncogene and whose overexpression is associated with tumorigenesis and progression, formation of multi-drug resistance (MDR), angiogenesis, invasion and metastasis. The role of COX-2 expression in epithelial ovarian cancer has been widely confirmed. It has been shown that COX-2 and ovarian cancer drug resistance were significantly correlated.

Naringin is a natural flavonoid drug with functions of anti-inflammation, anti-oxidant stress, blood glucose reduction, myocardial protection and anti-tumor effect [1]. Our preliminary study

has found that a certain dose of naringin can significantly inhibit the proliferation of drug-resistant ovarian cancer cells in vitro, and the effect of inhibition increases with increasing time and concentrations, and can reduce the expression of COX-2 protein [2]. The aim of the present study was to further investigate the potential mechanism of signaling pathway regulation by naringin, which induced inhibition of COX-2 transcriptional activity and reversed the cisplatin resistance of ovarian cancer cells, thus providing the experimental evidence for the development and application of naringin.

## Materials and methods

### Main reagents

Human epithelial ovarian cancer cell lines resistant to cisplatin (SKOV3/DDP) were purchased

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**Table 1.** The sequences of PCR primers and the product size

Gene name	Primer sequence (5'-3')	Product size (bp)
NF-κB	Forward primer: CAAGGAAGTCCCAGACCAAACC	121
	Reverse primer: CTCCTGCCCTACAGAGGTC	121
COX-2	Forward primer: CAAGCACTGTGGGTTTAAAT	273
	Reverse primer: GGTTTGTGTCAGCAGATCAAT	273
β-actin	Forward primer: CGGGAATCGTGCCTGAC	480
	Reverse primer: GGACTCGTCATACTCTGCT	480

from Shanghai Institute of Cell Chemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Naringin was given by Institute of Pharmacology at Nanchang University, China. Cisplatin (Shandong Qilu Pharmaceutical Co., Ltd.), RPMI-1640 medium, fetal bovine serum (Beijing Solarbio Science & Technology Co., Ltd.).

NF-κB, COX-2 antibodies (Abcam), horseradish peroxidase labeled goat anti-rabbit IgG (Jackson Immuno Research), TRITC-labeled goat anti-rabbit IgG (Beijing Zhongsheng Jinqiao Biotechnology Co., Ltd.) and Trizol kit (Invitrogen) were purchased. PCR primers were synthesized by Genscript Biotechnology Co. Ltd. (Table 1), and NF-κB siRNA and plasmids were constructed by Ribobio in Guangzhou.

### Experimental methods

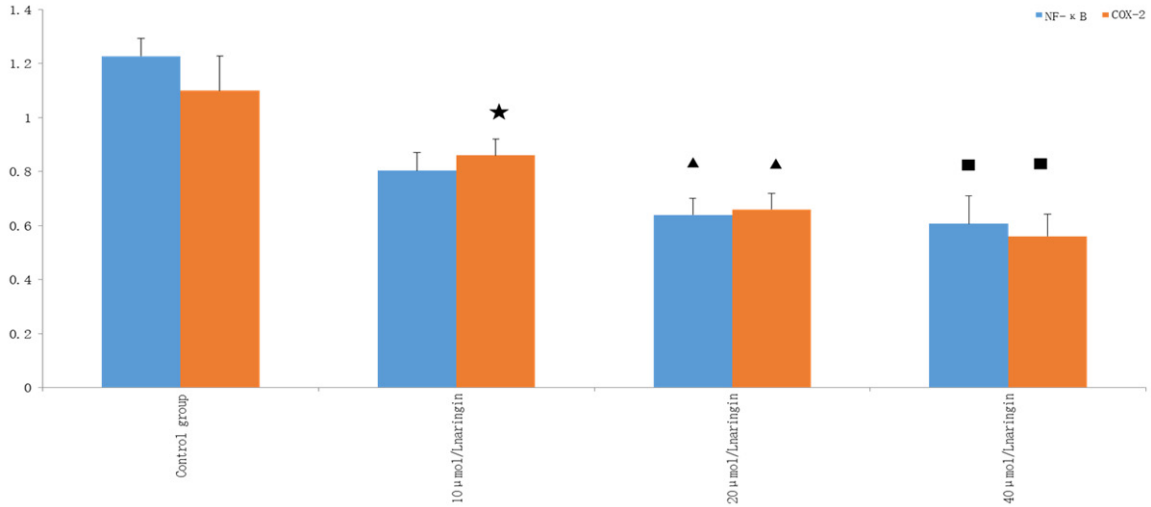
**Cell culture:** SKOV3/DDP cells were cultured in RPMI 1640 complete medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C under 5% CO<sub>2</sub> and saturated humidity. The medium was exchanged every day and the cells were passaged every 2-3 days. To maintain the drug resistance of SKOV3/DDP cell line to cisplatin, a low concentration of cisplatin (0.5 µg/ml) drug-containing culture medium was used for cell culture at 1 week before the experiment. The cells were randomly assigned to different groups after reaching the logarithmic growth phase: ① Normal control group: normal cultured SKOV3/DDP cells; ② Naringin treatment group: naringin was added into the medium at 10 µmol/L, 20 µmol/L and 40 µmol/L, respectively, and cultured for 48 h. Each group was done in 5 wells of duplicates. The experiment was repeated for 3 times and the mean was taken.

**Naringin preparation:** Four milligrams of naringin powder were accurately weighed and fully dissolved in 1 ml DMSO to make a 7 mmol/L naringin storage solution; under aseptic conditions, 200 µl of 7 mmol/L naringin storage solution was added into 6.8 ml of 1640 medium and fully mixed to make a 200 µmol/L naringin solution; 4 ml of 200 µmol/L naringin solution was diluted in 1640 culture medium to make naringin solution at concentrations of 40 µmol/L, 20 µmol/L and 10 µmol/L, respectively.

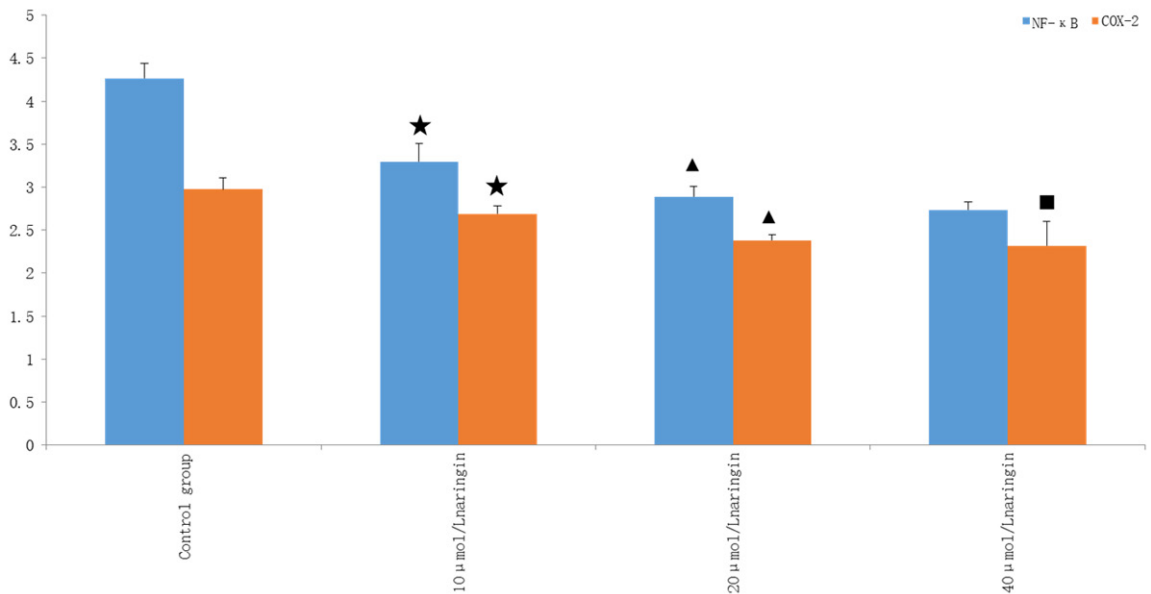
**RT-PCR analysis:** Total RNA was extracted from each of the above groups of cells, and cDNA was synthesized by reverse transcription according to the instructions of the reverse transcription kit DRR037A from TaKaRa. The PCR reaction conditions were as follows: reverse transcription at 37°C for 15 min and at 85°C for 5 s. The reaction conditions were as follows: pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 sec; annealing at 55°C for 30 sec; the cycles were repeated 29 times and followed by an extension step at 72°C for 30 sec; in the final step, the PCR products were extended at 72°C for 10 min. The PCR products were detected by chemiluminescence gel imaging system and the images were collected. The intensity of optical density (IOD) values of the target gene and the internal control β-actin band was analyzed by Gel-Pro software. The relative expression of the target genes in each group was compared.

**Western blot detection:** The cells were collected and protein lysis buffer was added to lyse the cells on ice. After centrifugation at 12000 r/min and 4°C for 15 min, the supernatant was collected and the protein concentration was determined by the BCA method. The concentration of each sample was adjusted to 2 µg/µl. After protein quantification, 40 µg of each sample underwent gel electrophoresis, membrane transfer, mounting and incubation with primary and secondary antibodies. The NC membrane was then taken for an ECL chemiluminescence reaction, and γ-tubulin was used as the internal reference to detect the bands, acquire the images and collect the results using a chemiluminescence gel imaging system. IPD6.0 soft-

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**Figure 1.** Effects of naringin at different concentrations on mRNA expression of NF-κB and COX-2 in SKOV3/DDP cell line. ★ $P < 0.05$ , compared to control group; ▲ $P < 0.05$ , compared to control group and 10 μmol/L naringin group, and  $P > 0.05$ , compared to 40 μmol/L naringin group; ■ $P < 0.05$ , compared to control group and 10 μmol/L naringin group, and  $P > 0.05$ , compared to 20 μmol/L naringin group.



**Figure 2.** The relative protein expression of NF-κB and COX-2 in SKOV3/DDP cell line at different concentrations of naringin. ★ $P < 0.05$ , compared to control group; ▲ $P < 0.05$ , compared to control group and 10 μmol/L naringin group, and  $P > 0.05$ , compared to 40 μmol/L naringin group; ■ $P < 0.05$ , compared to control group and 10 μmol/L naringin group, and  $P > 0.05$ , compared to 20 μmol/L naringin group.

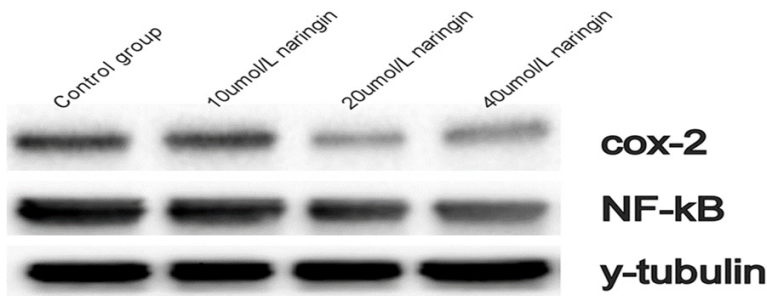
ware was used to analyze the IOD values of the bands of target proteins and the band of the internal reference protein  $\gamma$ -tubulin. A relative quantitative method was used to represent the expression level of the target proteins.

**Cell transfection and intervention:** Well-growing SKOV3/DDP cells in the logarithmic growth phase were inoculated in 6-well plates at  $2 \times 10^5$

cells per well, and were cultured at 37°C in a 5% CO<sub>2</sub> incubator until the cells reached a confluence of 70%-80% (after about 24 h). The cells were then transfected according to the transfection steps for Lipofectamine M2000 by Invitrogen.

The cells were divided into the following groups: blank control group (without plasmids and

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**Figure 3.** Effects of different concentrations of naringin on the expression of NF-κB and COX-2 proteins in SKOV3/DDP cell line.

**Table 2.** Overexpression and silencing of NF-κB on relative expression of COX-2 mRNA (x±s)

Group	COX-2
Blank control group	1.106±0.100
Empty plasmid control group	1.070±0.165
siRNA control group	1.023±0.105
NF-κB overexpression group	1.763±0.132★
NF-κB siRNA group	0.373±0.040◆
<i>F</i>	53.910
<i>P</i>	0.000

★*P*<0.05, compared to empty plasmid control group, blank control group; ◆*P*<0.05, compared to siRNA control group, blank control group.

siRNA), empty plasmid control group, NF-κB overexpression group, siRNA control group and NF-κB siRNA group. At 48 h after the transfection with NF-κB siRNA and the overexpression plasmids, the expression of COX-2 mRNA and protein were detected; the SKOV3/DDP cells transfected with the overexpression plasmids were treated with naringin in the following groups: naringin 20 µmol/L + NF-κB overexpression group and naringin 20 µmol/L + empty plasmid control group. The COX-2 mRNA and protein expression level were detected.

### Statistical methods

Statistical analysis was performed using the SPSS17.0 statistical software, and the measured data were expressed as mean ± standard deviations (mean ± s). The t test was used to compare the averages between two groups. The single factor analysis of variance (ANOVA) was used to compare among different groups. The LSD-t method was used to compare between two groups if the difference was sta-

tistically significant. *P* value <0.05 was considered statistically significant.

### Results

*Naringin suppressed NF-κB and COX-2 mRNA expression in SKOV3/DDP cells*

Both the results of RT-PCR and Western blot showed that there was significant difference between the two groups (*P*<0.05); and the expression of NF-κB and COX-2 decreased with an increase of naringin concentrations, and the difference was statistically significant (*P*<0.05) as compared to the control group; however, the difference between the 40 µmol/L and the 20 µmol/L groups was not statistically significant (*P*>0.05) (Figures 1-3).

### Overexpression and silencing of NF-κB on COX-2 mRNA expression

Both RT-PCR and WB detection found that the expression of COX-2 mRNA and protein in the NF-κB overexpression group were significantly higher while the expression of COX-2 mRNA and protein in the siNF-κB group was significantly lower, with significant difference compared to the values in the blank control group, the empty plasmid group and the siRNA control group (*P*<0.05) (Tables 2 and 3; Figures 2, 4, 5).

### Over-expression of NF-κB followed by naringin treatment on COX-2 mRNA and protein expression

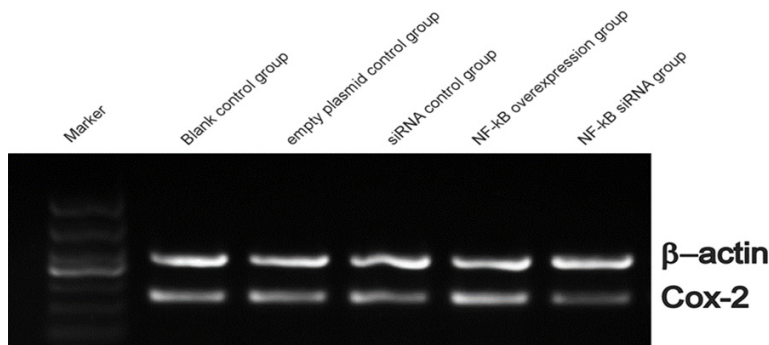
SKOV3/DDP cell in two groups with 20 µmol/L of naringin added. In experimental group, transfection was done with overexpression of NF-κB plasmid, and in control group, transfection was done with empty plasmid. WB results showed that the expression of NF-κB protein in experimental group was significantly higher than that in the control group, suggesting that transfection was successful (Figure 6A). RT-PCR test also showed that NF-κB mRNA increased compared with the control group (Figure 6B). After overexpression of NF-κB, the expression of mRNA and protein in COX-2 were higher than

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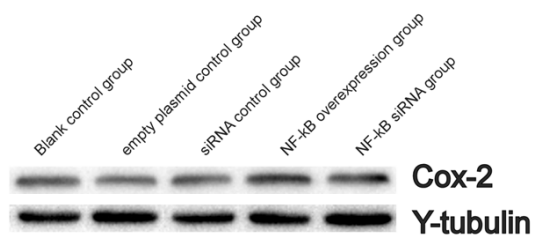
**Table 3.** Effect of NF- $\kappa$ B overexpression + naringin treatment on mRNA expression of P-gp

Group	COX-2	NF- $\kappa$ B
Naringin 20 $\mu$ mol/L + NF- $\kappa$ B overexpression group	0.940 $\pm$ 0.075	0.61 $\pm$ 0.089
Empty plasmid control group	1.257 $\pm$ 0.070★	1.797 $\pm$ 0.064◆
<i>P</i>	0.006	0.000

★*P*<0.05, compared the control group; ◆*P*<0.05, compared to the control group.



**Figure 4.** The expression level of COX-2 mRNA after overexpression and silencing of NF- $\kappa$ B.



**Figure 5.** The expression level of COX-2 mRNA after overexpression and silencing of NF- $\kappa$ B.

that in control group, the difference was statistically significant. (*P*<0.05) (**Figure 6C** and **6D**).

### Discussion

Epithelial ovarian cancer is the top killer of women's health. Tumor cytoreductive surgery and cisplatin-based chemotherapy are the main treatments of ovarian cancer [3]. However, chemotherapy resistance commonly seen in clinical applications has become a main reason for poor prognosis of ovarian cancer. With the molecular mechanism of chemotherapy resistance of ovarian cancer being investigated in-depth, corresponding molecularly targeted drugs that can reverse chemotherapy resistance and improve the sensitivity to the chemotherapy have gradually attracted the attention. However, the formation of drug resistance is a

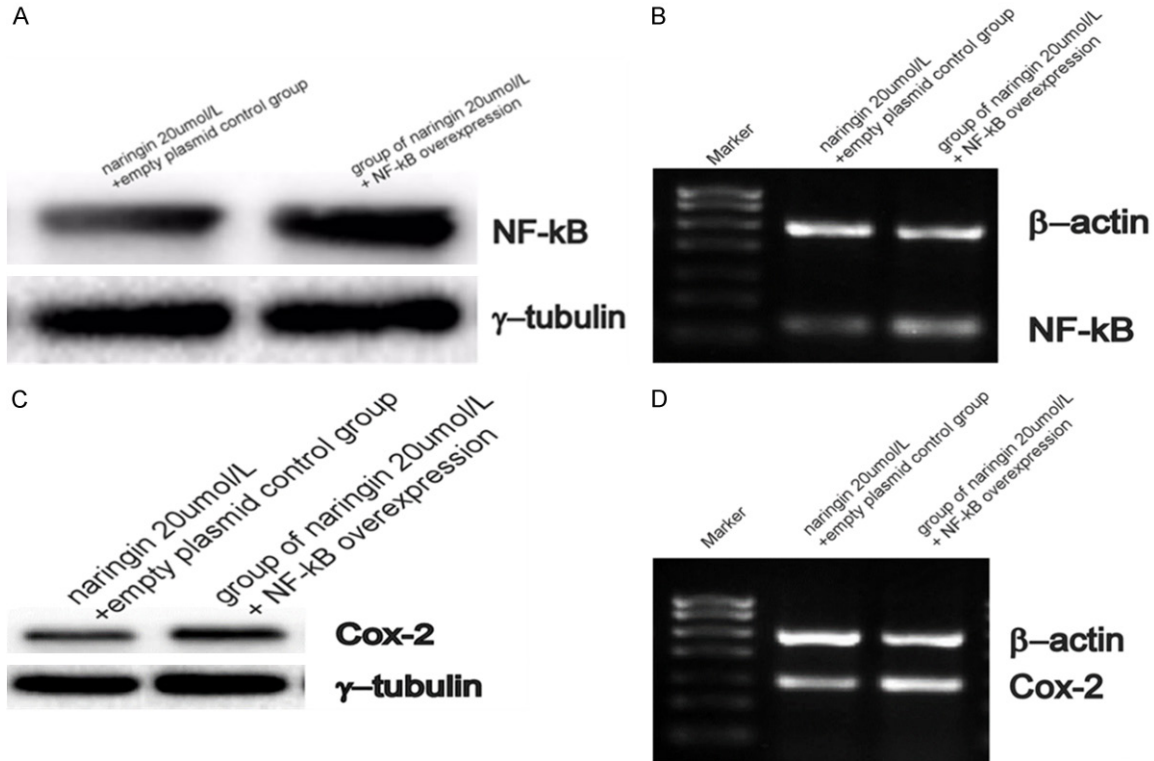
complex process involving multi-genes and multi-factors, and the non-specificity, side effects, and safety, economic and other issues related to the targeted therapy have limited its application. In recent years, some natural Chinese medicines have become a hot spot in the research field of drug resistance of ovarian cancer. Naringin, a natural flavonoid, has anti-inflammation, anti-oxidative stress, blood glucose reduction, myocardium

protection and anti-tumor effects. It shows small side effects and has good safety and economy values. It has been used in tumor therapy research, but its role in the mechanism of cisplatin resistance reversal of ovarian cancer has not been reported.

Our preliminary results suggest that naringin can down-regulate the expression of COX-2 protein in SKOV3 cells in vitro. Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase, is a key rate-limiting enzyme that catalyzes the synthesis of various prostaglandins from arachidonic acid. Multiple transcription factors and signaling pathways are involved in the regulation of COX-2 gene expression. Among them, the NF- $\kappa$ B signal pathway is a classical signaling pathway. The up-regulation of NF- $\kappa$ B can induce its expression in the cells. In contrast, the expression of COX-2 may be inhibited by interfering with the expression of p65, a down-stream gene in the NF- $\kappa$ B pathway.

COX-2 is an important oncogene involved in a variety of physiological and pathological processes. COX-2 expression in thyroid cancer, endometrial cancer, colon cancer, breast cancer, pancreatic cancer and other tumors is up-regulated [4-8]. Overexpression of COX-2 in epi-

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**Figure 6.** Expression of COX-2 and NF-κB between two groups. A: Expression of NF-κB protein in Naringin intervention + expression of NF-κB; B: Expression of NF-κB mRNA in Naringin intervention + expression of NF-κB; C: Expression of COX-2 protein in Naringin intervention + expression of NF-κB; D: Expression of COX-2 mRNA in Naringin intervention + expression of NF-κB.

thelial ovarian cancer is also widely recognized and involved in the development and progression of ovarian cancer [9, 10].

Recently, COX-2 has been shown to be significantly associated with tumor drug resistance in a way such that COX-2 can up-regulate the expression of multi-drug resistance proteins such as P-gp and GST-π, thus increasing tumor drug resistance and causing the failure of chemotherapy. COX-2-targeted inhibitors combined with chemotherapeutic agents have been shown to reverse tumor resistance and enhance the cytotoxic effects of chemotherapeutic agents [11, 12]. In ovarian cancer research, it has been shown that patients with high COX-2 expression had poor sensitivity to the chemotherapeutic agents [13]. In this study of ovarian cancer drug resistance, it was also found that naringin can inhibit the expression of COX-2 protein in vitro in the ovarian cancer drug-resistant cell line SKOV3/DDP, and the effect of naringin was concentration-dependent, confirming the inhibition effect of naringin on drug-resistant strains of ovarian cancer.

The study found that cisplatin-resistant ovarian cancer cells showed a continuously high expression of NF-κB, which played a core role in the drug resistance mechanism of ovarian cancer cells [14]. In our study, it was found that naringin inhibited the expression of NF-κB in SKOV3/DDP cells.

NF-κB signal pathway is closely related to the development of tumor. COX-2 is one of the downstream target proteins of the NF-κB signaling pathway. To confirm the relationship between NF-κB signal pathway and COX-2, we used a method to overexpress or silence the NF-κB in order to detect the expression of COX-2 mRNA before and after the intervention. The results showed that COX-2 mRNA in NF-κB overexpression group increased while the expression of COX-2 mRNA decreased in the siNF-κB group, confirming that COX-2 was regulated by the NF-κB signaling pathway. In the follow-up study, we added naringin as the intervention condition, and the results showed that by adding naringin, the COX-2 mRNA and protein expression in the NF-κB overexpression

group decreased as compared with that in the control group, so we speculate that naringin reverses the cisplatin drug resistance in ovarian cancer by a mechanism that is closely related to the regulation of COX-2 expression in SKOV3/DDP cell line through the NF- $\kappa$ B signaling pathway.

In conclusion, naringin can play a role in reversing the cisplatin resistance by inhibiting the expression of NF- $\kappa$ B and COX-2, and the role of COX-2 in the reversal process of drug resistance is mediated by NF- $\kappa$ B signaling pathway regulation. Therefore, some of the mechanisms played by naringin in the reversal of ovarian cancer platin-resistance are elucidated, and such findings provide the theoretical basis for the development and utilization of naringin, and also provide new ideas for the treatment of ovarian cancer.

### Disclosure of conflict of interest

None.

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