

***In vitro* study on reversal of ovarian cancer cell resistance to cisplatin by naringin via the nuclear factor- κ B signaling pathway**

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Abstract. The aim of the present study was to investigate the mechanism of action by which naringin reverses the resistance of ovarian cancer cells to cisplatin. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) and western blotting assays were used to detect the effects of different concentrations of naringin on the expressions of nuclear factor (NF)- κ B and P-glycoprotein (P-gp) in the SKOV3/CDDP cell line. Small interfering RNA (siRNA) targeting NF- κ B was designed and synthesized to silence NF- κ B, and recombinant plasmid vectors overexpressing NF- κ B were constructed to transfect cells. RT-qPCR and western blotting assays were subsequently performed to detect the effects of NF- κ B on the expression of P-gp at the mRNA and protein levels. Naringin was added to the NF- κ B-overexpressing SKOV3/CDDP cells and cultured for 48 h, followed by the detection of the expression of P-gp. RT-PCR and western blotting results demonstrated that the gene and protein expressions of NF- κ B and P-gp were significantly decreased in a dose-dependent manner by naringin treatment ($P < 0.05$). In cells overexpressing NF- κ B, P-gp expression was significantly elevated ($P < 0.05$), and the expression of P-gp was significantly decreased when NF- κ B was silenced ($P < 0.05$). Treatment with naringin was able to significantly ameliorate the NF- κ B-induced overexpression of P-gp ($P < 0.05$). These results indicate that naringin is able to inhibit the expression of NF- κ B and P-gp in SKOV3/CDDP

cells. Such an inhibitory effect may increase gradually with concentration, and is associated with blockade of the NF- κ B signaling pathway. This pathway may represent one of the mechanisms of action by which Naringin reverses resistance to platinum-based agents in ovarian cancer cells.

Introduction

Ovarian cancer is one of the three malignant tumors of female reproductive system. Statistics revealed that in 2014, there were ~21,980 new cases of ovarian cancer and ~14,270 patients succumbed to the disease in the United States (1). Due to the lack of specific symptoms in early stage of the disease and the lack of effective screening strategies, the majority of cases progress to an advanced stage at the time of primary diagnosis (1). As treatment is for ovarian cancer is long, painful and decreases patients' quality of life, and the recurrence rate is high, it was reported that 60-80% patients with advanced ovarian cancer relapse 2-3 years after the initial treatment (2) Therefore, ovarian cancer is regarded as the leading cause of mortality in female patients with a reproductive system malignant tumor (1). The most effective treatment for ovarian cancer is surgical cytoreduction and platinum-based chemotherapy (3). Although these treatments have improved the prognosis of patients to some extent, the 5-year survival rate of ovarian cancer remains at approximately 30-40% (4).

Resistance to platinum-based agents is one of the biggest factors affecting the therapeutic efficacy of pharmacological agents for ovarian cancer (5); therefore, identifying a highly efficient and low-toxic anti-cancer treatment that is able to attenuate resistance to platinum-based agents in ovarian cancer cells is of great importance. The Chinese medicine naringin, which is a natural flavonoid drug, has been demonstrated to have anti-cancer potential, with properties including inhibiting tumor cell proliferation, promoting tumor cell apoptosis and interfering in tumor cell signal transduction (6). A previous study by our group revealed that 20-40 μ mol/l naringin was able to significantly inhibit the proliferation of ovarian cancer cells resistant to platinum-based agents *in vitro* (7); however, the underlying mechanisms by which naringin reverses this resistance remain unclear. A previous study indicated that the nuclear factor (NF)- κ B signaling pathway serves a role in the

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development and progression of ovarian cancer (8). The aim of the present study is to investigate the mechanism of action by which naringin inhibits the expression of P-glycoprotein (P-gp) in a cisplatin-resistant human epithelial ovarian cancer cell line (SKOV3/CDDP) from the perspective of NF- κ B signal transduction, thereby providing an experimental rationale for the development and application of naringin as a treatment for ovarian cancer.

Materials and methods

Cells and reagents. The cisplatin-resistant ovarian cancer cell line SKOV3/CDDP was purchased from the Chinese Academy of Sciences (Beijing, China). Naringin was provided by the Institute of Pharmacology at Nanchang University (Nanchang, China), and cisplatin was purchased from Qilu Pharmaceutical Co., Ltd. (Shandong, China). RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from Beijing Soledad Lite-On Technology Co., Ltd. (Beijing, China). Antibodies directed against NF- κ B (cat. no. ab16502) and P-gp (cat. no. ab103477) were purchased from Abcam (Cambridge, UK), horseradish peroxidase-labeled (HRP) goat anti-rabbit IgG (cat. no. TA130015) was purchased from OriGene Technologies, Inc. (Beijing, China). TRIzol was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Primers for polymerase chain reaction (PCR) were synthesized by GenScript (Piscataway, NJ, USA). The NF- κ B small interfering (si)RNA (sense, 5'-GGAGUACCCUGAGGCUAUATT-3' and anti-sense, 5'-UAUAGCCUCAGGGUACUCCTT-3'), the negative control siRNA (sense, 5'-UUCUCCGAACGUGUCACGUCdTdT-3' and anti-sense, 5'-ACGUGACACGUUCGGAGAAAdTdT-3') and plasmid construction was performed by Guangzhou Ruibo Biological Technology Co., Ltd. (Guangzhou, China).

Experimental methods

Cell culture. SKOV3/CDDP cells were cultured in RPMI-1640 complete culture medium containing 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C in an atmosphere containing 5% CO₂, with the medium replaced every other day and passage performed every 2-3 days. Cells were cultured to the logarithmic phase and were then randomly assigned to either the normal control group (SKOV3/CDDP cells cultured in RPMI-1640 with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C and 5% CO₂) or the naringin treatment group (treated with 10, 20 or 40 μ mol/l naringin). Cells were cultured for 48 h at 37°C with or without naringin according to their group, with 5 duplicates performed for each group. All experiments were performed in triplicate.

Preparation of naringin. Naringin stock solution (7 mmol/l) was prepared by dissolving 4 mg naringin in 1 ml dimethylsulfoxide. Naringin solution (200 μ mol/l) was prepared by adding 200 μ l naringin solution to the RPMI-1640 culture medium (6.8 ml). Different concentrations of naringin solution (40, 20 and 10 μ mol/l) were prepared by diluting this solution with RPMI-1640 to different ratios.

Semi-quantitative reverse transcription (RT) PCR analysis. Total RNA was extracted from cells in the above groups using

TRIzol according to the manufacturer's protocol, and RNA was converted into cDNA using a reverse transcription kit (cat. no. DRR037A; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RT was performed using a thermocycler at 37°C for 15 min and at 85°C for 5 sec. Primer sequences for PCR are presented in Table I. Amplification was performed using 2xEasyTaq[®] PCR SuperMix (Beijing Transgen Biotech Co., Ltd., Beijing, China) and a thermocycler as follows: Pre-denaturation at 4°C for 5 min, then 29 cycles of denaturation at 94°C for 30 sec and annealing at 55°C for 30 sec, followed by extension at 72°C for 30 sec and 72°C for 10 min. The PCR products separated on a 2% agarose gel with ethidium bromide and detected using a chemiluminescent gel imaging system (ChemiDoc[™] XRS+; Bio-Rad, Hercules, CA, USA), and the Gel-Pro software (version 4.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to analyze the integrated optical density (IOD) values of the target gene bands.

Western blotting assay. Cells were collected and lysed with protein lysate on ice for 30 min at 4°C, followed by centrifugation 12,000 x g at 4°C for 15 min. The supernatant was collected and protein concentrations were determined using the bicinchoninic acid assay method, and each sample was diluted to a final concentration of 2 μ g/ μ l. A total of 8 μ g protein samples were loaded per lane and separated by a 4% (stacking) and 10% (resolving) SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, which was blocked with 5% skimmed milk for 2 h at room temperature. The membrane was then incubated with NF- κ B, P-gp and anti-tubulin (cat. no. T3526; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) primary antibodies overnight at 4°C, and incubated with the HRP-conjugated secondary antibodies for 2 h at room temperature. The nitrocellulose membrane was removed for visualization using Pierce[™] ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.); anti-tubulin was used as the internal control. IOD values of the bands were analyzed using Image Pro Plus software (version 6.0; Media Cybernetics, Inc.) and the relatively quantitative method [IOD (target protein)/IOD (reference protein) ratio] was used to detect the expression level of the target protein.

Cell transfection and intervention. When the SKOV3/CDDP cells reached 90-95% confluence, they were harvested and seeded in 6-well plates at a density of 2x10⁵ cells per well. Cells were cultured in an incubator at 37°C in an atmosphere containing 5% CO₂, until cells covered 70-80% of the plates (~24 h). Cells were subsequently transfected with 100 pmol plasmids or 100 pmol siRNA according to their group, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cells were divided into the following groups: Blank control group (no plasmids or siRNA), empty plasmid control group (plasmids without a gene), NF- κ B overexpression group (NF- κ B plasmids), siRNA control group (negative control siRNA) and the NF- κ B siRNA group (NF- κ B siRNA). The expression of P-gp mRNA was detected by semi-quantitative RT-PCR as previously mentioned at 48 h following transfection of NF- κ B siRNA or plasmid overexpression, and intervention with naringin was performed in SKOV3/CDDP cells

Table I. Primer sequences and product size.

Gene name	Primer sequence (5'-3')	Product size (bp)
Nuclear factor- κ B	F: CAAGGAAGTCCCAGACCAAACC R: CTCCTGCCCTACAGAGGTC	121
P-glycoprotein	F: TCAGTCAAGTTCAGAGTCTTCA R: CAAGGCAGTCAG TTACAGTCC	187
β -actin	F: CGGGAAATCGTG CGTGAC R: GGACTCGTCATA CTCCTGCT	480

F, forward; R, reverse.

Table II. Effects of different concentrations of naringin on the expression of nuclear factor- κ B and P-glycoprotein mRNA in the SKOV3/CDDP cell line.

Group	Nuclear factor- κ B	P-glycoprotein
Control	1.227 \pm 0.065	1.253 \pm 0.103
Naringin (10 μ mol/l)	0.803 \pm 0.067 ^a	0.907 \pm 0.031 ^a
Naringin (20 μ mol/l)	0.640 \pm 0.060 ^{a,b}	0.720 \pm 0.053 ^{a,b}
Naringin (40 μ mol/l)	0.607 \pm 0.102 ^{a,b}	0.660 \pm 0.060 ^{a,b}

Data are presented as the mean \pm standard deviation. ^aP<0.001 vs. control group; ^bP<0.05 vs. naringin (10 μ mol/l) group.

Table III. Relative amount of nuclear factor- κ B and P-glycoprotein proteins in the SKOV3/CDDP cell line with different concentrations of naringin.

Group	Nuclear factor- κ B	P-glycoprotein
Control group	4.267 \pm 0.172	3.323 \pm 0.097
Naringin (10 μ mol/l)	3.297 \pm 0.207 ^a	2.200 \pm 0.125 ^a
Naringin (20 μ mol/l)	2.890 \pm 0.120 ^{a,b}	1.847 \pm 0.093 ^{a,b}
Naringin (40 μ mol/l)	2.733 \pm 0.097 ^{a,b}	1.703 \pm 0.147 ^{a,b}

Data are presented as the mean \pm standard deviation. ^aP<0.001 vs. control group; ^bP<0.05 vs. naringin (10 μ mol/l) group.

transfected with NF- κ B overexpression plasmids. A previous study by our group demonstrated that, the growth rate of drug resistant cells was significantly decreased with a naringin dosage of 20 μ mol/l (7). Therefore, a working concentration of 20 μ mol/l naringin was used in the present study, and the groups used were as follows: Naringin (20 μ mol/l) + NF- κ B overexpression group and the empty plasmid control group. A total of 48 h after cell culturing, the expression of P-gp mRNA was detected by semi-quantitative RT-PCR as previously mentioned.

Statistical analysis. Statistical analysis was performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Student's t-test was used for comparisons of the mean values between the two groups. Intergroup comparisons were made using one-way analysis of variance, and the Fisher's least significant

differences t-test was further adopted for pairwise comparison. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of naringin on the expression of NF- κ B and P-gp at both mRNA and protein levels in SKOV3/CDDP cell lines. Semi-quantitative RT-PCR and western blotting results revealed that naringin significantly decreased the expression of NF- κ B and P-gp in a dose-dependent manner (P<0.05; Tables II and III; Fig. 1). Significant differences in NF- κ B and P-gp levels were observed between the 40 μ mol/l naringin and 10 μ mol/l naringin groups, and between the 20 μ mol/l naringin and 10 μ mol/l naringin groups (both P<0.05; Tables II and III; Fig. 1). However, there was no significant

difference between the 40 $\mu\text{mol/l}$ naringin and 20 $\mu\text{mol/l}$ naringin groups.

Effects of overexpression and silencing of NF- κ B on P-gp mRNA levels. RT-qPCR revealed that the expression of P-gp mRNA in the NF- κ B overexpression group was significantly upregulated compared with the blank control and empty plasmid control groups (both $P < 0.05$; Table IV; Fig. 2). However, P-gp expression was significantly downregulated in the siRNA NF- κ B group compared with the blank control and siRNA control groups ($P < 0.05$; Table IV; Fig. 2).

Effects of overexpression and silencing of NF- κ B and subsequent intervention with naringin. RT-qPCR results demonstrated that P-gp mRNA levels were significantly decreased in the NF- κ B overexpression group compared with the control group following intervention with naringin ($P < 0.05$; Table V; Fig. 3).

Discussion

Ovarian cancer is the primary cause of reproductive cancer-related mortality in women in the United States (1). Typically, symptoms do not present in the early stages of ovarian cancer, which renders it difficult to diagnose, and the majority of cases are at an advanced stage when diagnosed (9). At present, the suggested treatment methods of ovarian cancer are surgery and chemotherapy (10-13), and platinum-based agents and paclitaxel have become the first-line chemotherapeutic agents for the treatment of ovarian cancer (14,15). However, the emergence of multi-drug resistant ovarian cancer cells, on which chemotherapeutic agents are ineffective, may reduce the efficacy of chemotherapy (16). Various traditional Chinese medicines have demonstrated potential as anti-cancer agents, with advantages including no toxic reactions and the ability to reverse resistance to chemotherapeutic agents (17,18).

Naringin is a natural flavonoid compound that primarily exists in the peels of pomelo, grapefruit, lime and similar fruits (19). A previous study reported that Naringin exhibits anti-inflammatory, anti-oxidative stress, hypoglycemic, myocardial protective and anti-tumor effects (20). Naringin achieves its anti-tumor effects primarily via the inhibition of tumor cell proliferation, promoting tumor cell apoptosis and interfering in tumor cell signal transduction (21). The results of our previous study suggested that naringin is able to significantly inhibit the proliferation of SKOV3 and SKOV3/CDDP cells *in vitro* in a time- and dose-dependent manner (22). However, the mechanism by which naringin reverses resistance to platinum-based agents remains unclear. The results of the present study suggest that naringin is able to inhibit the expression of NF- κ B and P-gp proteins in the drug resistant ovarian cancer SKOV3/CDDP cell line *in vitro* in a concentration-dependent manner.

One important factor leading to chemotherapy resistance is drug efflux from cells. P-gp is an energy-dependent drug pump encoded by the multidrug resistance 1 (MDR1) gene, and its activation and expression are associated with a variety of signaling pathways, such as the phosphoinositide 3-kinase/protein kinase B, mitogen-activated protein kinase (MAPK), NF- κ B,

Table IV. Relative expression levels of P-glycoprotein mRNA following overexpression and silencing of NF- κ B.

Group	P-glycoprotein
Blank control	0.990 \pm 0.026
Empty plasmid control	1.056 \pm 0.085
siRNA control	1.030 \pm 0.020
NF- κ B overexpression	1.810 \pm 0.070 ^{a,b}
NF- κ B siRNA	0.437 \pm 0.095 ^{a,c}

Data are presented as the mean \pm standard deviation. ^a $P < 0.001$ vs. blank control group, ^b $P < 0.001$ vs. empty plasmid control group, ^c $P < 0.001$ vs. siRNA control group. NF- κ B, nuclear factor- κ B; siRNA, small interfering RNA.

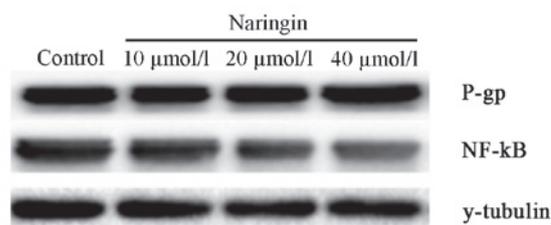


Figure 1. Effects of different concentrations of naringin on the expressions of NF- κ B and P-gp proteins in SKOV3/CDDP cell lines. NF- κ B, nuclear factor- κ B; P-gp, P-glycoprotein.

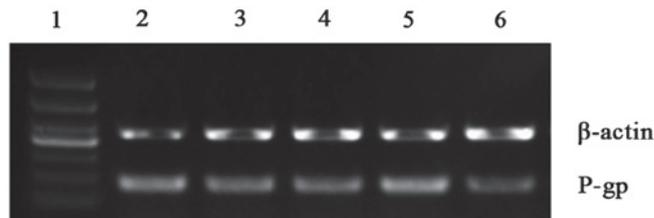


Figure 2. P-gp mRNA expression following the overexpression or silencing of NF- κ B. The lanes are as follows: 1, hyperladder; 2, blank control group; 3, empty plasmid control group; 4, siRNA control group; 5, NF- κ B overexpression group; 6, NF- κ B siRNA group. P-gp, P-glycoprotein; NF- κ B, nuclear factor- κ B; siRNA, small interfering RNA.

Wnt/ β -catenin Ras-Raf-MAPK kinase-extracellular signal-regulated kinase and prostaglandin E2-cAMP-protein kinase C-NF- κ B pathways (23). P-gp discharges multiple cytotoxic drugs using ATP within the cells, which increases the resistance of tumor cells to pharmacological agents, thereby causing the resistance of tumor cells (24). MDR1/P-gp is expressed in almost all human tumor cells to different degrees, and its expression in tumors is sensitive to chemotherapy or with poor efficacy is often high (25). Previous studies have reported that the sensitivity of some cancer patients to chemotherapy was increased following the administration of P-gp inhibitors (24,26-28). In the present study, downregulation of P-gp protein by naringin was also observed, and therefore it was inferred that the ameliorating effect of naringin on cisplatin resistance of ovarian cancer cells was associated with the downregulation of P-gp.

Table V. Effects of NF-κB over-expression and intervention with naringin on the expression of mRNA of P-gp.

Group	P-gp	NF-κB	T-value	P-value
Naringin (20 μmol/l) + NF-κB overexpression group	1.077±0.065 ^a	0.610±0.089 ^a	-8.496	0.001
Empty plasmid control group	1.753±0.080	1.797±0.064	-12.266	0.000

Data are presented as the mean ± standard deviation. ^aP<0.01 vs. control group. NF-κB, nuclear factor-κB; P-gp, P-glycoprotein.

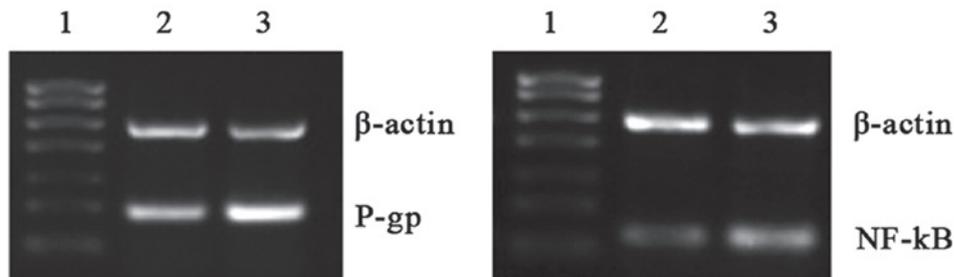


Figure 3. Effects of NF-κB overexpression + intervention with naringin on the expression of P-gp mRNA. The lanes are as follows: 1, hyperladder; 2, NF-κB overexpression cells treated with 20 μmol/l naringin; 3, empty plasmid control group. NF-κB, nuclear factor-κB; P-gp, P-glycoprotein.

The results of the present study indicate that naringin inhibits the expression of NF-κB in SKOV3/CDDP cells. Studies have reported that NF-κB is associated with the development, progression and metastasis of tumors, and many proteins it encodes promote tumor growth (29-31). One study demonstrated that NF-κB was persistently highly expressed in cisplatin-resistant ovarian cancer cells, and that it served an important role in drug resistance of ovarian cancer cells (5). NF-κB exerts anti-apoptotic effects via regulating downstream target proteins B-cell lymphoma (Bcl)-xL, Bcl-2, Fas/FasL, X-linked inhibitor of apoptosis protein, survivin, cellular inhibitor of apoptosis protein 1/2, cyclin-dependent kinase2, vascular endothelial growth factor and cyclooxygenase-2, whereby the viability of tumor cells is increased, resulting in chemotherapy resistance (8).

The NF-κB signaling pathway has been demonstrated by a number of studies to be associated with the development and progression of tumors (32,33). To further investigate the association between NF-κB and P-gp, which is an important gene contributing to drug resistance in ovarian cancer, the expression of P-gp mRNA was assessed before and after overexpression or silencing of NF-κB. The present results indicated that the expression of P-gp mRNA in the NF-κB overexpression group was increased, whereas it was decreased in the siRNA NF-κB group. This indicates that P-gp is subject to regulation by the NF-κB signaling pathway. In a later experiment, naringin was added as an intervention condition, and the expression of P-gp mRNA in the NF-κB overexpression group was decreased. These results suggest that the naringin-induced reversal of ovarian cancer resistance to platinum-based agents may be associated with the regulation of P-gp via the NF-κB signaling pathway in the SKOV3/CDDP cell line.

In conclusion, naringin is able to inhibit the expression of NF-κB and P-gp in SKOV3/CDDP cells. Such an inhibitory

effect may be dose-dependent, and is associated with the blockade of the NF-κB signaling pathway.

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