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Original Article

Apigenin induces apoptosis and reverses the drug resistance of ovarian cancer cells

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Abstract: Objective: Ovarian cancer is one of the most common malignant tumors in the female reproductive system and one of the most common gynecological diseases in the world. Its incidence has been increasing in recent years. Apigenin, an inexpensive drug, has few adverse reactions and significant anti-inflammatory, antiviral, and oxidative effects. If it can be applied to the treatment of ovarian cancer, it will have a great significance for future clinical treatment. The study examines the effect of Apigenin on apoptosis and the reversal of ovarian cancer cell drug resistance by regulating Mcl-1. Methods: The ovarian cancer-sensitive cell line SKOV3 and the ovarian cancer drug resistant cell line SKOV3/DDP were used as experimental samples. The ovarian cancer-sensitive cell line SKOV3 was divided into sensitive A (SKOV3+apigenin) with apigenin intervention, sensitive B (DPP+SKOV3) with DPP intervention, and sensitive C (apigenin+DDP+SKOV3) with apigenin and cisplatin (DDP) intervention. Similarly, the ovarian cancer drug resistant cell line SKOV3/DDP was divided into drug resistant A (SKOV3/DDP+apigenin), drug resistant B (SKOV3/DDP+DPP), and drug resistant C (SKOV3/DDP+DDP+SKOV3). The cell proliferation was tested using MTT in each group. The cell apoptosis in each group was tested using flow cytometry. The RNA and protein expressions of Mcl-1 were tested in each group. The anti-tumor effects of apigenin on the sensitive cell line and the drug resistant cell line were observed, as was the effects on the sensitivity of the various cell lines to the chemotherapeutic drug DDP. Results: There was no significant difference in the proliferation of cancer cells between sensitive A and sensitive B (P>0.050). The proliferation of sensitive C was significantly lower than the proliferation of sensitive A and sensitive B (P<0.050). There was no significant difference in the apoptosis between sensitive A and sensitive B (P>0.050). The apoptosis of sensitive C was significantly higher than the apoptosis of sensitive A and sensitive B (P<0.050). The cell proliferation in drug resistant A was significantly lower than it was in drug resistant B (P<0.050), and lower than it was in drug resistant C (P<0.001). There was no significant difference in apoptosis between drug resistant B and drug resistant C (P<0.050). The apoptosis of drug resistant A was significantly higher than the apoptosis of drug resistant B and C. Conclusion: Apigenin can enhance the chemosensitivity of ovarian cancer sensitive cells and drug resistant cells. The mechanism may work by inducing the apoptosis of ovarian cancer cells by down-regulating the Mcl-1 gene.

Keywords: Apigenin, Mcl-1 gene, ovarian cancer, SKOV3, SKOV3/DDP

Introduction

Ovarian cancer is one of the most common malignant tumors in the female reproductive system and one of the most common gynecological diseases in the world [1]. As shown in surveys, ovarian cancer patients have increased globally by an average of 300,000 people each year, the incidence of which shows a rising trend [2]. In addition, studies in recent years showed that more and more younger people suffer from ovarian cancer [3, 4]. If the current trend continues, ovarian cancer will be the most common malignant tumor among women within 30 years [5]. Moreover, ovarian cancer is a great threat, statistically, because the 5-year survival rate of patients with end-stage ovarian cancer is only 5.0%~30.0% [6]. With a high morbidity and a high risk, ovarian cancer has been the focus of research in clinical practice. However, the pathogenesis of ovarian cancer is still unclear. The main treatment is surgery combined with radiotherapy and chemotherapy [7]. With the development of diseases in

Table 1. Primer sequences

	GAPDH	Mcl-1
R	5'-ATCAAGAAGGTGGTGAAGCAG G-3'	5'-GAGGCTGGGATGGGTTTGTG-3'
F	5'-AAGGTGGAGGAGTGGGTG TCG-3'	5'-GGCTAGGTTGCTAGGGTGCA-3'

recent years, the shortcomings of traditional treatment have gradually become known. Clinically, the complex structure of tumors often leads to an incomplete excision or incomplete elimination of tumors. The recurrence and metastasis of ovarian cancer have also been greatly increased [8]. Therefore, more and more researchers at home and abroad attach importance to the targeted therapy of ovarian cancer. Apigenin is a flavonoid widely present in fruit, vegetables and beans [9]. At present, massive studies have proved that [9-11] apigenin plays vital anti-inflammatory, anti-viral, anti-oxidative roles and reduces blood pressure. With its low cost, infrequent adverse reactions, and strong curative effects, apigenin has a great potential for the treatment of ovarian cancer. However, the relationship between Apigenin and ovarian cancer has remained unclear. Therefore, this study provides a reference for the clinical application of apigenin in the treatment of ovarian cancer by observing the effect of apigenin on ovarian cancer cell lines.

Materials and methods

Source of cells

The ovarian cancer sensitive cell line SKOV3 was purchased from ATCC, USA, HTB-77, USA, and the ovarian cancer drug resistant cell line SKOV3/DDP was obtained from Shanghai Xin Yu Biotech Co., Ltd, XY0817.

Main reagents

The PCR Kit TransScript II Two-Step RT-PCR SuperMix and the total RNA extraction kit EasyPure PCR Purification Kit were used. Apigenin was purchased from the Wuhan Booute Biotechnology Co., Ltd., orb322340. Cisplatin (DPP) was purchased from Qingdao Jie Shi Kang Biotechnology Co., Ltd., 100401. The MTT Cell Proliferation and Cytotoxicity Assay Kit (MTT) was purchased from Shanghai Biyotime Biotechnology Co., Ltd., C0009. The RPMI-1640 medium, bovine fetal serum, trypsin, penicillin streptomycin solution, ECL chemiluminescent solution, RIPA, BCA protein kit, Annexin V-FITC Kit (China Shanghai Thermo Fisher Scientific Co., Ltd., 11875085, 12483020, A40007, 15070-063, 32209, 89900, 23250, 331200), Bax, Bcl-2, β-Actin, HRP-labeled Goat Anti-Mouse IgG (H+L) (R&D Company, AF820, AFD810, MAB8929, HAF008), flow cytometry (CytoFLEX, Beckman Coulter, USA). The primer sequence was designed by Shanghai Sangon Biotech Co., Ltd. (**Table 1**).

Cell culture

The SKOV3 cell line and the SKOV3/DDP cell line were cultured in RPMI-1640 medium (1% penicillin and streptomycin, 10% bovine fetal serum), then transferred to a 37°C, 5% CO_2 incubator to be cultured and generated.

Cell proliferation test

The 2nd to 3rd generation cells were collected and inoculated into 96 well plates at 1×10^4 cells/well (about 100 ugL). Two cell lines were divided into 4 groups, including sensitive A (Apigenin+SKOV3) and drug resistant A (Apigenin+SKOV3/DDP); sensitive B (DPP+SKOV3) and drug resistant B (DPP+SKOV3/DDP); sensitive C (Apigenin+DPP+SKOV3) and control group C (Apigenin+DPP9+SKOV3/DDP). The SKOV3 cells were used as the sensitive control group and the SKOV3/DDP cells were used as the drug resistant control group (treated without Apigenin and DPP but with the RPMI-1640 culture medium), and was transferred to a 5% CO_2 incubator at 37°C for 48 h. The cell proliferation in each group was tested according to the MTT at 24 h and 48 h, and the inhibition rate was calculated.

PCR test

The cells were tested using PCR and then transferred to a 5% CO_2 incubator for 48 hours. After that, the total RNA was extracted using an EasyPure PCR Purification Kit. The purity, concentration, and integrity of the total RNA were tested using an ultraviolet spectrophotometer and agarose gel electrophoresis. Reverse transcription of total RNA was performed by using 5× TransScript® II All-in-One SuperMix for PCR. The operation was done following the kit ma-

nufacturer's instructions. Next, the PCR amplification experiment was carried out. The reaction system was as follows: cDNA 2 μL, 1 μL of upstream and downstream primers, 2× TransScript® HIFI PCR SuperMix II 25 μL, Nuclease-free water was supplemented to 50 μL. The reaction conditions were as follows: 5 min for pre-denaturation at 94°C, 30 s for denaturation at 94°C, 30 s for annealing at 60°C, 45 s for extension at 72°C, with 40 cycles in total. Each sample has three repetition holes. The experiment was carried out three times. In this study, GAPDH was used as an internal reference. The data were analyzed using 2-Δct.

Western blot test

The cells were tested by Western blot. The total protein of cells was extracted using RIPA pyrolysis after 48 hours. The BCA protein determination method was used to detect the concentration. The protein concentration was adjusted to 4 μg/μL and separated using 12% SDS-PAGE electrophoresis. The membrane was transferred to a PVDF membrane, then dyed by ponceau and immersed in PBST for 5 min, then sealed with 5% skimmed milk powder for 2 h. Then the primary antibodies were added at 4°C overnight. The membrane was removed by getting the primary antibodies out. HRP (horseradish peroxidase) was added to label the goat anti-rat IgG (H+L)-HRP (1:5000), incubated at 37°C for 1 h and immersed in PBST three times for five minutes each time. The Images were processed in a darkroom. The excess liquid in the membrane was absorbed using filter paper. The light was given and developed using ECL. The protein bands were scanned and the gray values were analyzed with Quantity One, where the relative expression of the protein equals the gray value of the targeted protein band or the gray value of the β-Actin protein band.

Apoptosis test

The cultured cells were transferred to 15 ml tapered tubes and placed on ice after 48 h. The cells in the plate were washed with 2 mL PBS. Next, the PBS was removed while 0.5 ml 0.25% trypsin was added without EDTA. We observed the cells until they began to fall off from the culture plate under the microscope, the cells completely fell off from the culture plate wall through tapping. Resuspending the culture medium, we adjusted the cell density to 1×10^6 cells/ml and transferred 0.5 ml of the cell suspension to the clean centrifuge tube. Then 1.25 μl Annexin V-FITC was added and the temperature was kept at 18-24°C in the room and away from light for 15 min. Next we centrifuged the mixture at 1000 rpm for 5 min at room temperature to supernatant out. The cells were resuspended, and we added 10 μl propidium iodide and kept them on ice. Flow cytometry was used for the measurement and analysis.

Observation index

The proliferation and apoptosis of the cells were tested in each group. The expression of Mcl-1 was tested using PCR in each group. The expression levels of Mcl-1, Bax and Bcl-2 were tested using WB in each group.

Statistical methods

All the data were processed using SPSS 24.0 (Beijing Strong-Vinda Technology Co., Ltd.) software, and the images were plotted using GraphPad 8 (Shenzhen Softhead Technology Co., Ltd.). The results calculated by SPSS were checked twice. The results of this experiment were expressed as the means ± standard deviations. Independent samples t-tests were used for comparisons of the normal distribution data between two groups. An analysis of variance was used for the comparisons among multiple groups, and LSD-t tests were used for the comparisons between two groups after an analysis of variance. $P<0.050$ was considered statistically significant.

Results

Cell proliferation test

Tested by MTT, the cell inhibition rates of the sensitive A, sensitive B, sensitive C, and the sensitive control groups were (9.29±1.50)%, (15.67±2.84)%, (24.62±3.96)%, 0% respectively. Among them, there was no inhibition in the control group, and the inhibition rate of the other three groups was significantly higher than it was in the sensitive control group ($P<0.050$). The sensitive A was significantly lower than the sensitive B and sensitive C ($P<0.050$). The sensitivity C was the highest ($P<0.050$). The cell inhibition rates of drug resistant A, drug resistant B, drug resistant C, and the drug

Table 2. Cell inhibition expressions of the SKOV3 cell line (%)

Group	Inhibition rate
Sensitive control	0
Sensitive A	9.29±1.50[a]
Sensitive B	15.67±2.84[a,b]
Sensitive C	24.62±3.96[a,b,c]

Note: a indicates a comparison with the sensitive control group, $P<0.050$; b indicates a comparison with sensitive A, $P<0.050$; c indicates a comparison with sensitive C, $P<0.050$.

Table 3. Cell inhibition expressions of the SKOV3/DPP cell line (%)

Group	Inhibition rate
Drug resistance control	0
Drug resistant A	14.86±2.05[a]
Drug resistant B	0.42±0.06[a,b]
Drug resistant C	28.66±3.82[a,b,c]

Note: a indicates a comparison with the drug resistant control group, $P<0.050$; b indicates a comparison with drug resistant A, $P<0.050$; c indicates a comparison with drug resistant C, $P<0.050$.

Table 4. The expressions of apoptosis in the SKOV3 cell line (%)

Group	Apoptotic rate
Sensitive control	2.56±1.08
Sensitive A	12.62±2.61[a]
Sensitive B	18.47±3.92[a,b]
Sensitive C	29.35±4.06[a,b,c]

Note: a indicates a comparison with the sensitive control group, $P<0.050$; b indicates a comparison with sensitive A, $P<0.050$; c indicates a comparison with sensitive C, $P<0.050$.

Table 5. The expressions of apoptosis in the SKOV3/DPP cell line (%)

Group	Apoptotic rate
Drug resistance control	3.52±1.83
Drug resistant A	18.41±3.11[a]
Drug resistant B	5.96±1.24[a,b]
Drug resistant C	26.93±5.26[a,b,c]

Note: a indicates a comparison with the drug resistant control group, $P<0.050$; b indicates a comparison with drug resistant A, $P<0.050$; c indicates a comparison with drug resistant C, $P<0.050$.

resistant control groups were (14.86±2.05)%, (0.42±0.06)%, (28.66±3.82)%, and 0%, respectively. Among them, there was no inhibition in the control group, and the inhibition rate of the other three groups was significantly higher than it was in the drug resistant control group ($P<0.050$). The drug resistant A was significantly higher than the drug resistant B ($P<0.050$) but lower than that of drug resistant C ($P<0.050$) (**Tables 2** and **3**).

Cell apoptosis test

Flow cytometry showed that the apoptosis rates of sensitive A, sensitive B, sensitive C, and the sensitive control group were (12.62±2.61)%, (18.47±3.92)%, (29.35±4.06)%, 0% respectively. Among them, the apoptosis rates of sensitive A, B, and C were significantly higher than the rate of the sensitive control group ($P<0.050$); the sensitive B was significantly higher than the rate of sensitive A ($P<0.050$), sensitive C was significantly higher than the rate of sensitive B ($P<0.050$). The apoptosis rates of drug resistant A, drug resistant B, drug resistant C, and the drug resistant control group were (18.41±3.11)%, (5.96±1.24)%, (26.93±5.26)%, and (3.52±1.83)% respectively. Among them, the apoptosis rates of drug resistant A, B, and C were significantly higher than the rate of the drug resistant control group ($P<0.050$), and drug resistant B was significantly lower than the rate of drug resistant A and C ($P<0.050$), and drug resistant C was significantly higher than the rate of drug resistant A ($P<0.050$) (**Tables 4** and **5**).

The expression of mRNA

The expression of mRNA in each group was determined using PCR. The results showed that the expressions of mRNA in sensitive A, sensitive B, sensitive C, and the sensitive control group were 0.63±0.20, 0.58±0.12, 0.28±0.09, 0.92±0.13 respectively. The expressions of mRNA of sensitive A, B and C was significantly lower than the expressions in the sensitive control group ($P<0.050$). There was no significant difference in the expression of mRNA between sensitive A and sensitive B ($P>0.050$), but sensitive C was significantly lower than the expressions in sensitive A and B ($P<0.050$). The expressions of mRNA in drug resistant A, drug resistant B, drug resistant C, and the drug resistant control group were 0.59±0.12, 1.10±0.09, 0.22±0.07 and 1.18±0.08 respectively. There was no significant difference in the expressions of mRNA between drug resistant B and the drug resistant control group ($P>0.050$), but drug resistant A and drug resis-

Table 6. The expressions of Mcl-1 mRNA in the SKOV3 cell line

Group	Mcl-1 mRNA
Sensitive control	0.92±0.13
Sensitive A	0.63±0.20[a]
Sensitive B	0.58±0.12[a]
Sensitive C	0.28±0.09[a,b,c]

Note: a indicates a comparison with the sensitive control group, *P*<0.050; b indicates a comparison with sensitive A, *P*<0.050; c indicates a comparison with sensitive C, *P*<0.050.

Table 7. The expressions of Mcl-1 mRNA in the SKOV3/DPP cell line

Group	Mcl-1 mRNA
Drug resistance control	1.18±0.08
Drug resistant A	0.59±0.12[a]
Drug resistant B	1.10±0.09[b]
Drug resistant C	0.22±0.07[a,b,c]

Note: a indicates a comparison with the drug resistant control group, *P*<0.050; b indicates a comparison with drug resistant A, *P*<0.050; c indicates a comparison with drug resistant C, *P*<0.050.

Table 8. The expressions of the Mcl-1 protein in the SKOV3 cell line

Group	Mcl-1
Sensitive control	1.08±0.12
Sensitive A	0.70±0.04[a]
Sensitive B	0.56±0.09[a,b]
Sensitive C	0.32±0.05[a,b,c]

Note: a indicates a comparison with the sensitive control group, *P*<0.050; b indicates a comparison with sensitive A, *P*<0.050; c indicates a comparison with sensitive C, *P*<0.050.

Table 9. The expressions of the Mcl-1 protein in the SKOV3 cell line

Group	Mcl-1
Drug resistance control	0.98±0.06
Drug resistant A	0.46±0.09[a]
Drug resistant B	0.95±0.08[b]
Drug resistant C	0.20±0.10[a,b,c]

Note: a indicates a comparison with the drug resistant control group, *P*<0.050; b indicates a comparison with drug resistant A, *P*<0.050; c indicates a comparison with drug resistant C, *P*<0.050.

tant C was significantly lower than the expressions in drug resistant B and in the drug resistant control group (*P*<0.050). Drug resistant A had a significantly higher expression than drug resistant C did (*P*<0.050) (**Tables 6** and **7**).

Expression of Mcl-1

The expression of Mcl-1 in each group was tested using western blot. The results showed that the expressions of Mcl-1 in sensitive A, sensitive B, sensitive C and the sensitive control group were 0.70±0.04, 0.56±0.09, 0.32±0.05, and 1.08 ± 0.12 respectively. The expression of Mcl-1 in sensitive A, B, and C was significantly lower than it was in the sensitive control group (*P*<0.050), but the sensitive B expression was lower than it was in sensitive A (*P*<0.050), and the sensitive C expression was significantly lower than it was in sensitive A and B (*P*<0.050). The expressions of Mcl-1 in drug resistant A, drug resistant B, drug resistant C, and in the drug resistant control group were 0.46±0.09, 0.95±0.08, 0.20±0.10 and 0.98±0.06 respectively. There was no significant difference in the expressions of Mcl-1 between drug resistant B and the drug resistant control group (*P*>0.050), but the drug resistant A and drug resistant C expressions were significantly lower than they were in drug resistant B and in the drug resistant control group (*P*<0.050), and the expression in drug resistant A was significantly higher than it was in drug resistant C (*P*<0.050) (**Tables 8** and **9**).

Discussion

Ovarian cancer is the most common malignant tumor in female patients. The lack of any obvious symptoms in the early stage may mislead patients. Most patients found to be suffering from it are in the middle and late stages when metastasis and invasion occur, making the treatment and prognosis more difficult [12, 13]. The incomplete surgical removal of a tumor can affect patient rehabilitation [14]. At present, the commonly used chemoradiotherapy drugs such as cisplatin (DPP) and adriamycin are good at killing cancer cells, but they also have a bad impact on normal cell metabolism [13]. Therefore, an effective treatment for ovarian cancer with fewer side effects is urgently needed.

As a kind of flavonoid compound with multiple biological activities, apigenin has been shown to inhibit various tumor cells. And Bao et al. [15] showed that Apigenin could inhibit the

proliferation of ovarian cancer CAOV3 cells, but research on apigenin and SKOV3 cells is still limited. And the outdated research of Bao et al. [16] showed that apigenin could inhibit the proliferation of CAOV3 cells, but the effect of apigenin on ovarian cancer cells is still unknown. And Shi et al. [17] suggested that apigenin could block the growth cycle of colon cancer cells by regulating the expression of cyclin D1 to promote the activity of CDK1. Some studies also showed that the Mcl-1 gene has an impact on cyclin D1 [18]. Apigenin may affect the biological behavior of ovarian cancer cells through Mcl-1 based on the analysis from our experiment.

Treated with apigenin, the proliferation and apoptosis of the SKOV3 cells and SKOV3/DPP cells were significantly reduced, indicating that Apigenin has a strong effect on ovarian cancer cells. Apoptosis, the most effective way to treat tumors, plays a role in promoting the apoptosis of tumors and reducing their proliferation [19]. This study found that apigenin has the potential to be a targeted therapy for ovarian cancer. And the proliferation and apoptosis of SKOV3 cells and SKOV3/DPP cells treated with Apigenin and DPP were further compared to show the effect is much better than treatment with Apigenin only. Apigenin can increase the sensitivity of SKOV3/DPP cells to cisplatin to produce the effect of drug resistance. At present, the mechanism of acquiring chemotherapeutic resistance to tumors has not been completely clarified. And the failure of chemotherapy is that the increase of the anti-apoptosis of tumors is caused by the resistance to chemotherapy drugs [20]. In this study, apigenin, combined with cisplatin, was found to enhance the transformation of the SKOV3/DPP cells from phase G0/G1 to S, thereby improving the apoptosis of cancer cells.

In order to understand the apigenin mechanism on ovarian cancer cells, the levels of Mcl-1 were tested using PCR and Western blot in each group. The results showed that the mRNA and protein expressions of Mcl-1 were lower in the group with higher proliferation inhibition and apoptosis rates, which is consistent with the results of Polier et al. [21]. It is suggested that apigenin may induce the apoptosis of ovarian cancer cells and inhibit the proliferation of ovarian cancer cells by decreasing the expression of Mcl-1. The Mcl-1 gene, a kind of apoptosis control gene, combines with Bal-2 to synthesize competing homologous (heterologous) dimers [22]. Synthetic factors promote the release of apoptotic factors through the influx of Ca^{2+} [23]. Apigenin may change the permeability of the mitochondrial membrane by down-regulating the expression of Mcl-1, which prevents the combination of the apoptotic factors with cells, thereby improving the apoptosis of tumors. And Xiucong et al. [24] found that apigenin induced the apoptosis of SKOV3 cells by restraining the cell cycle at G2/M. Baradaran et al. [25] suggested that down-regulating the Mcl-1 gene can enhance diallyl disulfide (DADS) on leukemia HL-60 cell proliferation by blockage at G2/M, so the cells at G2/M are closely related to Mcl-1. However, Xiucong et al. [25] did not provide details on Mcl-1, so more experiments are needed to prove the result.

In this study, apigenin was found to inhibit the proliferation and apoptosis of SKOV3 cells and SKOV3/DPP cells in vitro. Some shortcomings exist due to the limited experimental conditions. For example, speculation suggests that apigenin may affect the biological behavior of cells by down-regulating the expression of Mcl-1 to change the permeability of the mitochondrial membrane, but the mitochondria in the cells have not been detailed. Moreover, no other apoptosis-related factors but the mRNA and protein expressions of Mcl-1 were considered in this study. At present, the effects and mechanisms of apigenin in the diagnosis of ovarian cancer cells are not clear. We hope to conduct more clinical trials in the future to confirm this study.

In conclusion, apigenin can enhance the chemosensitivity of ovarian cancer sensitive cells and drug resistant cells. The mechanism may involve inducing the apoptosis of ovarian cancer cells by down-regulating the Mcl-1 gene.

Disclosure of conflict of interest

None.

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