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Mechanism of Metformin Regulating Drug Resistance in Ovarian Cancer Cells through Parkin mediated P53 Ubiquitination

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summary

Objective: To explore the molecular mechanism of metformin regulating drug resistance in ovarian cancer cells through Parkin mediated P53 ubiquitination, and to provide new targets and experimental evidence for drug resistance therapy in ovarian cancer.Method: Sixty ovarian cancer patients who visited our hospital from January 2022 to December 2023 were selected, and their cancer tissue and adjacent normal tissue specimens were collected.Human ovarian cancer cell lines SKOV3 and drug-resistant SKOV3/DDP were cultured in vitro and divided into control group (untreated), metformin group (treated with 20 mmol/L metformin), Parkin overexpression group (transfected with Parkin overexpression plasmid), Parkin silencing group (transfected with Parkin siRNA), and metformin+Parkin silencing group (transfected with Parkin siRNA and added with 20 mmol/L metformin).Western blot was used to detect the expression levels of Parkin, P53, Poly-Ub, P-gp, and MRP1 in each group of cells;Immunoprecipitation assay was used to detect the interaction between Parkin and P53, as well as the ubiquitination level of P53;The CCK-8 method was used to detect the half maximal inhibitory concentration (IC50) ofcisplatin (DDP) in each group of cells;Flow cytometry was used to detect the apoptosis rate of cells in each group.Result: The expression level of Parkin in ovarian cancer tissues was significantly lower than that in adjacent normal tissues ($P<0.05$), while the expression levels of P53, P-gp, and MRP1 were significantly higher than those in adjacent normal tissues ($P<0.05$).Compared with SKOV3 cells, Parkin expression was significantly reduced ($P<0.05$), while P53, P-gp, and MRP1 expression were significantly increased ($P<0.05$) in SKOV3/DDP cells. IC50 for DDP was significantly increased ($P<0.05$), and apoptosis rate was significantly reduced ($P<0.05$).After treatment with metformin, Parkin expression in SKOV3/DDP cells significantly increased ($P<0.05$), while P53, P-gp, and MRP1 expression significantly decreased ($P<0.05$), P53 ubiquitination levels significantly increased ($P<0.05$), IC50 for DDP significantly decreased ($P<0.05$), and apoptosis rate significantly increased ($P<0.05$).The expression of P53, P-gp, and MRP1 in cells overexpressing Parkin was significantly lower than that in the control group ($P<0.05$), and the level of P53 ubiquitination was significantly higher than that in the control group ($P<0.05$). The IC50 for DDP was significantly reduced ($P<0.05$), and the apoptosis rate was significantly increased ($P<0.05$);The Parkin silence group showed the opposite trend ($P<0.05$).There was no significant difference in the above indicators between the metformin+Parkin silencing group and the Parkin silencing group ($P>0.05$).The immunoprecipitation results showed that Parkin interacts with P53, and treatment with metformin can enhance their binding ability ($P<0.05$).Conclusion: Metformin can upregulate the expression of Parkin, promote the interaction between Parkin and P53, and degrade P53 ubiquitination, thereby reducing the expression of resistance related proteins P-gp and MRP1, reversing the resistance of ovarian

cancer cells to cisplatin, suggesting that Parkin may be a potential target for drug-resistant treatment of ovarian cancer.

keywords

Metformin;Parkin；P53；Ubiquitination;oophoroma;drug resistance

Preface

Ovarian cancer is one of the common malignant tumors in the female reproductive system. Its incidence rate ranks the third among gynecological malignant tumors, but its mortality rate ranks the first, seriously threatening women's life and health [1].At present, surgery combined with platinum based chemotherapy is the main treatment option for ovarian cancer, among which cisplatin (DDP) is a commonly used first-line chemotherapy drug in clinical practice.However, as chemotherapy progresses, about 70% of patients will develop chemotherapy resistance, leading to treatment failure and tumor recurrence, which is a major challenge faced in the treatment of ovarian cancer [2].Therefore, in-depth exploration of the mechanism of drug resistance in ovarian cancer and search for effective strategies to reverse drug resistance are of great significance for improving the treatment efficacy and survival rate of ovarian cancer patients.

Metformin is a commonly used metformin based hypoglycemic drug in clinical practice. In recent years, research has found that it has potential anti-tumor effects, which can inhibit the proliferation, invasion, and metastasis of tumor cells through various mechanisms, and can reverse the chemotherapy resistance of tumor cells [3].Parkin is an E3 ubiquitin ligase belonging to the Parkin like protein family, initially extensively studied for its association with familial Parkinson's disease.In recent years, studies have shown that Parkin is abnormally expressed in various malignant tumors and is involved in the occurrence, development, and drug resistance regulation of tumors [4].P53 is an important tumor suppressor gene, and its functional abnormalities are closely related to the occurrence and development of tumors.In tumor cells, the expression level and activity of P53 are strictly regulated by the ubiquitination proteasome pathway, and Parkin, as an E3 ubiquitin ligase, may be involved in the ubiquitination degradation process of P53 [5].

This study speculates that metformin may reverse the drug resistance of ovarian cancer cells by regulating the expression of Parkin, thereby mediating the ubiquitination degradation of P53 and affecting the expression of resistance related proteins.To verify this hypothesis, this study investigated the effect and molecular mechanism of metformin on drug resistance in ovarian cancer cells by detecting the expression of Parkin, P53 and other related proteins in ovarian cancer tissues and cells, aiming to provide new theoretical basis and therapeutic targets for the treatment of ovarian cancer drug resistance.

1. Data and Methods

1.1 General Information

Sixty ovarian cancer patients who underwent surgical treatment in the obstetrics and gynecology department of our hospital from January 2022 to December 2023 were selected as the research subjects.Inclusion criteria: (1) Diagnosed with ovarian cancer through histopathological examination;(2) Not receiving chemotherapy, radiation therapy, or other anti-tumor treatments before surgery;(3) Complete clinical data.Exclusion criteria: (1) Combined with other malignant tumors;(2) Severe dysfunction of important organs such as the heart, liver, and kidneys;(3) Pregnant or lactating women.60 patients were aged 35-68 years, with an average age of (51.2 ± 6.8) years;Pathological types: 42 cases of serous cystadenocarcinoma, 10 cases of mucinous cystadenocarcinoma, and 8 cases of endometrioid

carcinoma;FIGO staging: 22 cases in stages I-II and 38 cases in stages III-IV.Collect cancer tissue samples from all patients and normal tissue samples adjacent to the cancer at a distance of≥ 5 cm from the edge of the cancer tissue. After sample collection, immediately freeze and store them in liquid nitrogen for future use.This study was approved by the Medical Ethics Committee of our hospital, and all patients signed informed consent forms.

Cell lines: human ovarian cancer cell line SKOV3 and cisplatin resistant SKOV3/DDP were purchased from the Chinese Academy of Sciences Shanghai Cell Bank.Main reagents: Metformin (Sigma, USA), Cisplatin (Qilu Pharmaceutical Co., Ltd., China), Parkin overexpression plasmid, Parkin siRNA and negative control siRNA (Shanghai Jima Pharmaceutical Technology Co., Ltd., China), Lipofectamine 3000 transfection reagent (Invitrogen, USA), CCK-8 kit (Biyuntian Biotechnology Research Institute, China), Annexin V-FITC/PI apoptosis detection kit (Nanjing Kaiji Biotechnology Development Co., Ltd., China), rabbit anti human Parkin antibody, rabbit anti human P53 antibody, rabbit anti human Poly Ab antibody, rabbit anti human P-gp antibody, rabbit anti human MRP1 antibody, horseradish peroxidase labeled goat anti rabbit IgG secondary antibody (Abcam, UK), immunoprecipitation kit (Thermo Fisher Scientific, USA).Main instruments: CO2 incubator (Thermo Fisher Scientific, USA), inverted microscope (Olympus, Japan), Western blot electrophoresis and membrane transfer instrument (Bio Rad, USA), flow cytometer (BD, USA), ELISA reader (Thermo Fisher Scientific, USA).

1.2 Method

1.2.1 Cell culture and grouping

SKOV3 and SKOV3/DDP cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin, and incubated in a constant temperature incubator at 37 ℃ and 5% CO2.Conduct the experiment when the cells reach the logarithmic growth phase.Divide SKOV3/DDP cells into 5 groups: control group (without any treatment), metformin group (treated with metformin at a final concentration of 20 mmol/L for 48 hours), Parkin overexpression group (transfected with Parkin overexpression plasmid for 48 hours), Parkin silencing group (transfected with Parkin siRNA for 48 hours), and metformin+Parkin silencing group (transfected with Parkin siRNA for 24 hours and then cultured with metformin at a final concentration of 20 mmol/L for 24 hours).The transfection operation should be carried out according to the Lipofectamine 3000 transfection reagent manual.

1.2.2 Western blot detection of related protein expression

Collect cell and tissue samples from each group, add RIPA lysis buffer (containing protease inhibitor) to ice for 30 minutes, centrifuge at 4 ℃ and 12000 r/min for 15 minutes, and extract total protein.The protein concentration was determined using the BCA method. The protein sample was mixed with the loading buffer and boiled at 100 ℃ for 5 minutes for denaturation.Take an equal amount of protein (30 μ g) for SDS-PAGE electrophoresis, and transfer the protein onto a PVDF membrane after electrophoresis.5% skim milk was sealed at room temperature for 2 hours, and primary antibodies (Parkin, P53, Poly Ab, P-gp, MRP1 antibody dilution ratio of 1:1000) were added and incubated overnight at 4 ℃.Wash the membrane with TBST three times, each time for 10 minutes. Add horseradish peroxidase labeled secondary antibody (1:5000) and incubate at room temperature for 1 hour. Wash the membrane with TBST three times, each time for 10 minutes.The ECL chemiluminescence assay kit was used for development, and ImageJ software was used to quantitatively analyze the grayscale values of the bands. Using β - actin as an internal reference, the grayscale value ratio of the target protein to β - actin was calculated, which is the relative expression level of the target protein.

1.2.3 Immunoprecipitation detection of Parkin P53 interaction and P53 ubiquitination level

Collect SKOV3/DDP cells from the control group and metformin group, add immunoprecipitation lysis buffer (containing protease inhibitor) and lyse on ice for 30 minutes, centrifuge at 4 ℃ and 12000 r/min for 15 minutes, and collect the supernatant.Take a portion of the supernatant as input, add rabbit anti human Parkin antibody (1 μ g) to the remaining supernatant, incubate overnight at 4 ℃, add Protein A/G agarose beads, and incubate at 4 ℃ for 2 hours. Centrifuge at 4 ℃ and 3000 r/min for 5 minutes, discard the supernatant, wash the agarose beads three times with lysis buffer, add loading buffer, and boil at 100 ℃ for 5 minutes.Western blot was used to detect the expression of P53 and Poly Ub in the precipitate, with Input as the positive control.

1.2.4 Detection of Cell IC50 for Cisplatin by CCK-8 Method

Inoculate SKOV3/DDP cells of logarithmic growth stage from each group into a 96 well plate at a density of 5 × 103 cells/well, with a volume of 100 μ L per well. After 24 hours of cultivation, add different concentrations of cisplatin (0, 2.5, 5, 10, 20, 40 μ mol/L) to each well. Set up 3 wells at each concentration and continue to cultivate for 48 hours. Add 10 μ L CCK-8 solution to each well, incubate at 37 ℃ for 2 hours, and measure the absorbance (OD value) at 450 nm wavelength using an enzyme-linked immunosorbent assay (ELISA) reader.Calculate cell survival rate: Cell survival rate (%)=(experimental group OD value - blank group OD value)/(control group OD value - blank group OD value) × 100%.Use GraphPad Prism 8.0 software to fit the dose-response relationship curve and calculate the IC50 of cisplatin in each group of cells.

1.2.5 Flow cytometry detection of cell apoptosis rate

Inoculate SKOV3/DDP cells from each group at a density of 2 × 105 cells/well into a 6-well plate, culture for 48 hours, then add cisplatin at a final concentration of 10 μ mol/L and continue to culture for 24 hours. Collect the cells, wash them twice with PBS, resuspend them in 100 μ L Binding Buffer, add 5 μ L Annexin V-FITC and 5 μ L PI, incubate at room temperature in the dark for 15 minutes, add 400 μ L Binding Buffer, and detect cell apoptosis rate using flow cytometry. Repeat the experiment three times.

1.3 Evaluation Criteria

Relative protein expression level: represented by the gray value ratio of the target protein detected by Western blot to the internal reference protein β - actin.Cell resistance to cisplatin: expressed as IC50 value, the larger the IC50 value, the stronger the cell's resistance to cisplatin.Apoptosis rate: expressed as the percentage of Annexin V-FITC positive and PI negative cells detected by flow cytometry to the total cells.

1.4 Statistical indicators

SPSS 26.0 statistical software was used for data analysis.The measurement data is expressed as mean ± standard deviation (x ± s), and t-test or one-way ANOVA is used for inter group comparisons, while LSD-t test is used for multiple comparisons.The count data is expressed as a rate (%), and the comparison between groups is conducted using the chi square test.A difference of $P<0.05$ is considered statistically significant.

2 results

2.1 Expression of related proteins in ovarian cancer tissue and adjacent normal tissue

Western blot analysis showed that the relative expression level of Parkin protein in ovarian cancer tissues was significantly lower than that in normal tissues adjacent to the cancer ($P<0.05$), while the relative expression levels of P53, P-gp, and MRP1 proteins were significantly higher than those in normal tissues adjacent to the cancer ($P<0.05$).The specific results are shown in Table 1.

Organization alt type	count down	Parkin	P53	P-gp	MRP1
Normal tissue adjacent to cancer	60	0.82±0.15	0.35±0.08	0.28±0.06	0.31±0.07

Ovarian cancer tissue	60	0.376±0.09	0.795±0.00	0.878±0.11	0.241
T-value	-	20.347	25.723	8.679	5.235
P-value	-	<0.001	<0.001	<0.001	<0.001

Note: Compared with normal tissue adjacent to cancer,P<0.05

2.2 Comparison of related protein expression and drug resistance between SKOV3 and SKOV3/DDP cells

Compared with SKOV3 cells, the relative expression level of Parkin protein in SKOV3/DDP cells was significantly reduced (P<0.05), while the relative expression levels of P53, P-gp, and MRP1 proteins were significantly increased (P<0.05);The IC50 of SKOV3/DDP cells to cisplatin was significantly higher than that of SKOV3 cells (P<0.05), and the apoptosis rate was significantly lower than that of SKOV3 cells (P<0.05).The specific results are shown in Table 2.

cell line	Parkin	P53	P-gp	MRP1	IC50;μm	Apoptosis

e						ol/L;	israte(%)
SKOV3	0.076±0.019	0.042±0.007	0.035±0.009	0.038±0.008	82.55±0.23	1	28.65±3.12
SKOV3/DDP	0.029±0.007	0.083±0.011	0.032±0.013	0.085±0.012	25.68±2.35	2	10.32±1.85
T-value	14.876	16.923	17.254	12.615	23.689		19.874
Pvalue	<0.001	<0.001	<0.001	<0.001	<0.001		<0.001

Note: Compared with SKOV3 cells,P<0.05

2.3 Effects of different treatments on SKOV3/DDP cell related indicators

Compared with the control group, the relative expression levels of Parkin protein in SKOV3/DDP cells were significantly increased in the metformin group and Parkin overexpression group (P<0.05), while the relative expression levels of P53, P-gp, and MRP1

proteins were significantly decreased (P<0.05). The ubiquitination level of P53 was significantly increased (P<0.05), and the IC50 for cisplatin was significantly reduced (P<0.05), while the apoptosis rate was significantly increased (P<0.05);The Parkin silence group showed the opposite trend (P<0.05).There was no significant difference in the above indicators between the metformin+Parkin silencing group and the Parkin silencing group (P>0.05).The specific results are shown in Table 3.

group	Parkin	P53	P-gp	MRP1	P53ubiquitination	IC50;μmol/L;	Apoptosis rate(%)
control group	0.30±0.08	0.82±0.09	0.91±0.11	0.84±0.14	0.25±0.10	26.12±2.41	10.56±1.92
Metformin group	0.68±0.10	0.45±0.09	0.42±0.08	0.45±0.09	0.78±0.13	10.35±1.56	26.89±3.05
P	0	0	0	0	0	9	2

arkinoverexpressiongroup	7.72±0.10	4.43±0.00	4.43±0.00	8.81±0.00	7.87±0.14	1±0.42	7.56±3.18
Parkinson Silence Group	0.12±0.00	1.12±0.00	1.22±0.00	1.10±0.00	8.65±3.24	3±0.03	5.23±1.05
Metformin+	0.15±0.05	1.12±0.01	1.25±0.01	1.18±0.01	1.28±0.04	9±3.16	7.68±2.12

Parkin silencing group							
F value	128.654	112.345	135.789	126.543	142.876	156.985	138.456
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Compared with the control group,P<0.05； Compared with the Parkin silence group, # P>0.05

2.4 Interaction between Parkin and P53 and the effect of metformin on it

The immunoprecipitation results showed that Parkin interacts with P53 in SKOV3/DDP cells;Compared with the control group, the binding ability of Parkin and P53 in metformin group cells was significantly enhanced (P<0.05), and the ubiquitination level of P53 was significantly increased (P<0.05).

3 Discussion

Chemotherapy resistance in ovarian cancer is the main cause of treatment failure, and its mechanism of occurrence is complex, involving the regulation of multiple genes and signaling pathways [6].In recent years, more and more research has focused on the role of metabolic drug metformin in anti-tumor and reversing chemotherapy resistance.This study explored the mechanism of metformin regulating drug resistance in ovarian cancer cells through Parkin mediated P53 ubiquitination through clinical specimen detection and in vitro cell experiments, providing new ideas for the treatment of ovarian cancer drug resistance [7].

This study first detected the expression of related proteins in cancer tissues and adjacent normal tissues of 60 ovarian cancer patients. The results showed that Parkin expression was significantly reduced in ovarian cancer tissues, while P53, P-gp, and MRP1 expression were significantly increased.This suggests that Parkin may play an anti-cancer role in the occurrence and development of ovarian cancer, while P53, P-gp, and MRP1 may be involved in the progression of ovarian cancer.P-gp and MRP1 are important members oftheATP binding cassette (ABC) transporter family, which can actively pump chemotherapy drugs out of cells, reduce intracellular drug concentrations, and lead to drug resistance in tumor cells [8].Therefore, the high expression of P-gp and MRP1 in ovarian cancer tissue may be one of the important reasons for its development of chemotherapy resistance [9].

To further investigate the relationship between Parkin and drug resistance in ovarian cancer, this study compared the differences in relevant indicators between the ovarian cancer cell line SKOV3 and the drug-resistant strain SKOV3/DDP.The results showed that compared with SKOV3 cells, Parkin expression was significantly reduced in SKOV3/DDP cells, while P53, P-gp, and MRP1 expression were significantly increased. IC50 for cisplatin was significantly increased, and apoptosis rate was significantly reduced.This indicates that the low expression of Parkin may be closely related to the development of drug resistance in ovarian cancer cells, suggesting that Parkin may be involved in the regulation of drug resistance in ovarian cancer cells [10].

Metformin, as a potential anti-tumor drug, has attracted much attention for its mechanism of reversing tumor resistance.This study found that metformin treatment significantly upregulated the expression of Parkin in SKOV3/DDP cells, reduced the expression of P53, P-gp, and MRP1, increased the ubiquitination level of P53, reduced the IC50 of cells to cisplatin, and increased the apoptosis rate [11].This suggests that metformin may promote the ubiquitination degradation of P53 by upregulating Parkin expression, thereby reducing the expression of resistance related proteins and reversing the drug resistance of ovarian cancer cells [12].To verify the role of Parkin reversing drug resistance with metformin, this study conducted Parkin overexpression and silencing experiments.The results showed that overexpression of Parkin can mimic the effect of metformin, reduce the expression of P53, P-gp, and MRP1, increase the level of P53 ubiquitination, and enhance the sensitivity of cells to cisplatin;And Parkin silencing can counteract the effect of metformin, restoring the above indicators to levels close to the control group.This further confirms the crucial role of Parkin reversing drug resistance in ovarian cancer cells with metformin [13].

The ubiquitination proteasome pathway is an important mechanism for regulating protein degradation, with E3 ubiquitin ligase playing a crucial role in specific recognition of substrate proteins.Parkin, as an E3 ubiquitin ligase, can mediate its ubiquitination degradation by binding to substrate proteins.This study confirmed through immunoprecipitation experiments that Parkin interacts with P53, and treatment with metformin can enhance their binding ability and increase the ubiquitination level of P53.This suggests that metformin may enhance Parkin's ubiquitination degradation of P53 by promoting the binding of Parkin to P53, thereby reducing the expression level of P53.P53, as an important transcription factor, its overexpression can affect the drug resistance of tumor cells by regulating the expression of downstream target genes [14].In this study, the expression level of P53 was positively correlated with the expression levels of resistance related proteins P-gp and MRP1, suggesting that P53 may upregulate the expression of P-gp and MRP1, leading to the development of drug resistance in ovarian cancer cells.Therefore, metformin mediated the degradation of P53 ubiquitination through Parkin, reducing the expression of P53 and subsequently downregulating the expression of P-gp and MRP1, which may be an important molecular mechanism for its reversal of drug resistance in ovarian cancer cells [15].

In summary, this study confirms that metformin can upregulate the expression of Parkin, promote the interaction between Parkin and P53, and degrade P53 ubiquitination, thereby reducing the expression of resistance related proteins P-gp and MRP1 and reversing the resistance of ovarian cancer cells to cisplatin.This discovery not only reveals a new

mechanism by which metformin reverses drug resistance in ovarian cancer, but also provides new potential targets for the treatment of ovarian cancer drug resistance [16].However, this study is only an in vitro experiment, and its results need to be further validated in animal models and clinical studies.In the future, we will delve into the clinical application value of metformin and Parkin in the treatment of ovarian cancer, providing more theoretical basis for personalized treatment of ovarian cancer patients.

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