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MicroRNA 1301 inhibits cisplatin resistance in human ovarian cancer cells by regulating EMT and autophagy

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Abstract. – OBJECTIVE: Ovarian cancer is prone to chemoresistance, leading to poor outcomes in patients. MicroRNA 1301 plays a regulatory role in multiple tumors. However, whether microRNA 1301 regulates cisplatin resistance in ovarian cancer cells remains unclear.

MATERIALS AND METHODS: The ovarian cancer SKOV3 cell line and the human ovarian cancer cisplatin-resistant strain cell SKOV3/DDP were cultured in vitro and microRNA1301 expression was analyzed by Real time PCR. MicroRNA1301 mimics and microRNA 1301 were transfected into SKOV3/DDP, respectively followed by analysis of cell proliferation by MTT assay, cell invasion, expression of autophagy genes ATG5 and Beclin1 and EMT-related transcription factors Snail and Slug by Real time PCR, expression of NF-κB and E-cadherin and N-cadherin by Western blot.

RESULTS: MicroRNA 1301 expression was significantly increased in SKOV3/DDP cells compared with that in SKOV3 cells ($p<0.05$). MicroRNA1301 mimics transfection into SKOV3/DDP up-regulated microRNA1301 expression, promoted cell proliferation, and invasion, inhibited ATG5 and Beclin1 expression, and promoted Snail and Slug expression, decreased E-cadherin expression and increased N-cadherin and NF-κB expression, compared with the control group, the differences were statistically significant ($p<0.05$). MicroRNA1301 inhibitor transfection into SKOV3/DDP cells could down-regulate the expression of microRNA1301 and significantly reversed the above changes. Compared with the control group, differences were statistically significant ($p<0.05$).

CONCLUSIONS: Targeting microRNA1301 can inhibit the proliferation of cisplatin-resistant cells and the development of EMT in human ovarian cancer cells by inhibiting the NF-κB signaling pathway, thereby inhibiting the occurrence and development of drug-resistant ovarian cancer.

Key Words:

Ovarian cancer, Drug resistance, Cisplatin, MicroRNA1301, NF-κB, Autophagy, Proliferation, EMT.

Introduction

Ovarian cancer (OC) is one of the malignant gynecological tumors. The incidence rate is in the top three of gynecological tumors. The mortality rate is high, and the prognosis is poor. The 5-year survival rate is only around 25-30%[1,2]. Ovarian cancer is considered to be the world's highest mortality fatal gynecological cancer, and epithelial ovarian cancer (EOC) accounts for 90% of OC cases in Asian populations[3]. Although the treatment of ovarian cancer is progressing day by day, ovarian cancer is prone to chemotherapy resistance, resulting in poor outcomes. In clinical studies, early metastasis and chemotherapy resistance lead to poor prognosis of EOC[4]. EOC treatment is mainly surgery, and platinum-based chemotherapy such as cisplatin is used as an auxiliary tumor cytoreductive surgery[5,6]. According to statistical analysis, approximately 15% to 25% of EOC patients have primary resistance to platinum-based chemotherapy, and at least 80% of patients eventually develop tumor recurrence[7]. The drug resistance mechanism in ovarian cancer is complex and has not yet been elucidated. Multidrug resistance-related genes and protein products, lung resistance-related protein (LRP), and glutathione transferase (GST), can induce drug efflux and inactivation, activation of DNA damage response pathway (DDR), abnormalities of drug target or apoptosis[8-10]. Therefore, it is urgent to uncover the molecular mechanism for drug resistance in ovarian cancer.

MicroRNAs (miRNAs) are important components of epigenetic research, with a wide range of regulation, diverse regulatory pathways, and target specificity[11,12]. MiRNAs are short-chain non-coding RNAs of small molecules with less than 22 nucleotides in length. Abnormal expression of miRNAs is involved in many disease

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processes and is closely related to tumorigenesis, tumor progression, and drug resistance[13,14]. Studies have confirmed that abnormally expressed microRNAs are involved in the regulation of ovarian cancer resistance and some miRNA expression up-regulation can inhibit the development of ovarian cancer drug-resistant cells; while other miRNA over-expression can promote the development of ovarian cancer drug-resistant cells, playing a promoting role[15]. MicroRNA 1301 plays a regulatory role in a variety of tumors[16]. However, whether microRNA 1301 regulates cisplatin resistance in ovarian cancer cells and related mechanisms has not been fully elucidated.

Materials and Methods

Main Instruments and Reagents

Ovarian cancer SKOV3 cell line and human ovarian cancer cisplatin-resistant strain cell SKOV3/DDP were preserved in our laboratory and stored in liquid nitrogen. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and cyan chain double antibody were purchased from HyClone (South Logan, UT, USA). Dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT) powder was purchased from Gibco (Grand Island, NY, USA); trypsin-ethylenediaminetetraacetic acid EDTA digest was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from Pall Life Sciences (Port Washington, NY, USA), EDTA was purchased from HyClone (South Logan, UT, USA), Western blot related chemical reagents were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China), enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK), rabbit anti-human NF-κB monoclonal antibody, rabbit anti-Human E-cadherin and N-cadherin mAb, mouse anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell signaling technology (Danvers, MA, USA). The transwell chamber was purchased from Corning (Corning, NY, USA). RNA extraction kit, reverse transcription kit, and lipo2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). TaqMan MicroRNA Reverse Transcription Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). MicroRNA1301 mimics and microRNA 1301 inhibitor were synthesized by Shanghai Jima Pharmaceutical Technology Co., Ltd (Shanghai, China). Other commonly used reagents were purchased from Shanghai Shenggong Biological Co., Ltd (Shanghai, China). Ultra-clean workbench was purchased from Suzhou Sutai Purification Equipment Engineering Co., Ltd (Suzhou, China). Thermo Scientific Forma CO_2 incubator was purchased from Thermo Fisher Scientific (Waltham, MA, USA). ABI 7700 Fast Quantitative PCR Reactor was purchased from ABI (Waltham, MA, USA).

Ovarian Cancer SKOV3 Cell Line, Human Ovarian Cancer Cisplatin-Resistant Strain Cell SKOV3/DDP and Grouping

Ovarian cancer cell lines SKOV3 and SKOV3/DDP were stored in liquid nitrogen, thawed in a 37°C water bath, centrifuged at 1000 rpm for 3 min, resuspended in 1 ml of fresh medium, transferred to a 5 ml cell culture flask containing 2 ml of fresh medium, and incubated for 24-48 h in a 37°C 5% CO_2 incubator. SKOV3/DDP cells were seeded in culture dishes at 1×10^6 cells/cm^2, including 10% FBS, 90% high glucose DMEM medium (containing 100 U/ml penicillin, 100 μg/ml streptomycin), and cultured in 37°C 5% CO_2 incubator. The experiment used 3-8 generation logarithmic growth phase cells. SKOV3/DDP was divided into three groups, control group, miR-1301 inhibitor group, and miR-1301 group, which was transfected with miR-1301 inhibitor and miR-1301 mimics in SKOV3/DDP cells, respectively.

Liposome Transfection of MiR-1301 Inhibitor and MiR-1301 Mimics in SKOV3/DDP Cells

MiR-1301 inhibitor and miR-1301 mimics were transfected into SKOV3/DDP cells, respectively. The miR-1301 inhibitor sequence was 5'-CGCAUAUGUGAGUGGCUA-3'. The miR-1301 mimics sequence was 5'-CAGUGCGAUAUGUGGCUA-3'. The cell density was fused to 70-80%. MiR-1301 inhibitor and miR-1301 mimics liposomes were separately added to 200 μl of serum-free medium, mixed well, and incubated at room temperature for 15 min. The mixed lipo2000 was mixed with miR-1301 inhibitor or miR-1301 mimics dilution and incubated for 30 min at room temperature. The serum of the cells was removed, PBS was gently rinsed, 1.6 ml of serum-free medium was added, and each system was added to each system, and cultured in a 5%

CO_2 incubator at 37°C for 6 h. The serum culture solution was replaced and cultured for 48 h for experimental research.

Real-Time PCR Detection of MiR-1301, ATG5 and Beclin1, and Snail and Slug Expression in SKOV3/DDP Cells

RNA from each group of SKOV3/DDP cells was extracted with TRIzol reagent, and DNA reverse transcription synthesis was performed according to the kit instructions. The primers were designed by Primer Premier 6.0 according to each gene sequence and synthesized by Shanghai Yingjun Biotechnology Co., Ltd. (Table I). Real-time PCR reaction conditions: 55°C 1 min, 92°C 30 s, 58°C 45 s, 72°C 35 s, a total of 35 cycles. Data was collected using the PCR reactor software and GAPDH was used as a reference. According to the fluorescence quantification, the starting cycle number (CT) of all samples and standards was calculated. Based on the standard CT value, a standard curve was drawn and then the semi-quantitative analysis was carried out by the 2-ΔCt method to calculate the expression of miRNA.

MTT Assay to Detect the Proliferation of Cells In Each Group

SKOV3/DDP cells collected in logarithmic growth phase were inoculated into the 96-well culture plate with 10% fetal bovine serum DMEM medium in the number of 5×10^3 cells. After 24 h of culture, the supernatant was discarded and cells in each group were tested at intervals of 24 h. 20 μl of sterile MTT was added to the well, and 3 replicate wells were set at each time point. After 4 h of continuous culture, the supernatant was completely removed, 150 μl/well of DMSO was added, and the shaken was shaken for 10 min until the purple crystals were fully dissolved. The absorbance (A) value was measured at 570 nm to calculate the proliferation rate of each group.

Western Blot Analysis of NF-κB and E-cadherin and N-cadherin Protein Expression Changes

SKOV3/DDP cell proteins were extracted from each group: the lysate was added, the cells were lysed on ice for 15-30 min, the cells were disrupted by sonication for 5 s × 4 times, centrifuged at 4°C, 10 000 × g for 15 min, and the supernatant was transferred to a new Eppendorf (EP) tube. In the tube, the protein was quantified and stored at -20°C for Western blot experiments. The isolated protein was electrophoresed on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry transfer method, 100 mA, 1.5 h and blocked with 5% skim milk powder for 2 h to remove non-specific background. After that, the membrane was incubated with primary antibody 1:1000, 1:1500, 1:2000 diluted NF-κB, and E-cadherin and N-cadherin monoclonal antibody at 4°C overnight. After washing with phosphate-buffered saline and tween-20 (PBST), 1:2000 diluted goat anti-rabbit secondary antibody was added and incubated for 30 min, followed by washing with PBST, addition of chemiluminescence for 1 min, and subsequent X-ray exposure imaging. X-film and strip density measurements were separately scanned using protein image processing system software and Quantity one software. The experiment was repeated four times (n=4).

Transwell Chamber Analysis of Cell Invasion

The serum-free medium was replaced according to the kit instructions. After 24 h, the bottom and membrane upper chambers were coated with a 1:5 50 mg/L Matrigel dilution and air dried at 4°C. 100 μl of tumor cell suspension prepared by adding 10 μl of FBS DMEM medium and serum-free medium were added to the chamber, and 3 replicate wells were set in each group. The chamber was placed in a 24-well plate. Each

Table I. Primer sequences.

Gene	Forward 5′-3′	Reverse 5′-3′
GAPDH	AGTACCGTCTAGTCTGG	TAATAGAATGTCGGCTGGT
MiR-1301	CTCACATACAGTGTCCCAG	GGGATCCGATTGTCCATGATT
ATG5	CTTACCGACCATGTCTGCC	CCGGTACTCACTGTCTCCTT
Beclin1	ATGCTTCTCCTAAGTTGG	TCGTCTTACCGCCCACA
Snail	TCACATACTCCTCCGA	ACTCCCTAAGTACCCATG
Slug	CAGTACCCGTCCTC	GTACTCGATTCTGCCTTACCG

control group was cultured in a transwell chamber without Matrigel. After 48 h of culture, the transwell chamber was washed with PBS, the cells on the membrane were removed and fixed in ice ethanol. After staining with crystal violet, the cells in the lower layer of the microporous membrane were counted. The experiment was repeated three times.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Comparison of the difference between two groups was assessed by *t*-test test and analyzed by SPSS 11.5 statistical software (SPSS Inc., Chicago, IL, USA). The differences between groups were analyzed by ANOVA with Bonferroni post-hoc test and the count data were analyzed by Chi-square test. $p < 0.05$ was considered a statistically significant difference.

Results

Expression of MicroRNA1301 In Human Ovarian Cancer Cisplatin Resistant SKOV3/DDP Cells

The expression of microRNA1301 in human ovarian cancer cisplatin resistant SKOV3/DDP cells was analyzed by Real time PCR and found that the expression of microRNA1301 was increased in SKOV3/DDP cells, and the difference was statistically significant compared with SKOV3 cells ($p < 0.05$; (Figure 1).

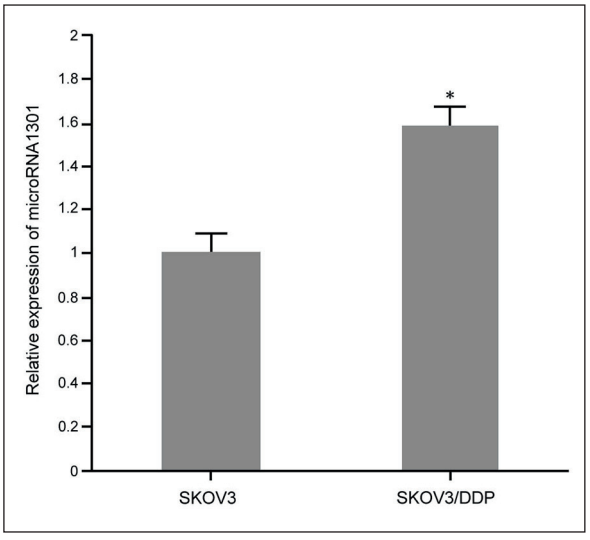

Figure 1. Expression of microRNA1301 in human ovarian cancer cisplatin resistant SKOV3/DDP cells. Compared with SKOV3 cells, *$p < 0.05$.

Effect of MicroRNA1301 on the Expression of MicroRNA1301 In SKOV3/DDP Cells

The effects of miR-1301 inhibitor and miR-1301 mimics on the expression of microRNA1301 in SKOV3/DDP cells were observed. Results showed that microRNA1301 mimics transfection into SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells and compared with the control group, the difference was statistically significant ($p < 0.05$). The transfection of miR-1301 inhibitor into SKOV3/DDP cells resulted in a decrease in the expression of miR-1301. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 2).

Effect of MicroRNA1301 on Proliferation of SKOV3/DDP Cells

MTT assay was used to analyze the effect of microRNA1301 on the proliferation of SKOV3/DDP cells. Transfection of microRNA1301 mimics into SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells, which further promoted cell proliferation. The difference was statistically significant ($p < 0.05$). Transfection of miR-1301 inhibitor into SKOV3/DDP cells decreased the expression of miR-1301 and inhibited cell proliferation. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 3).

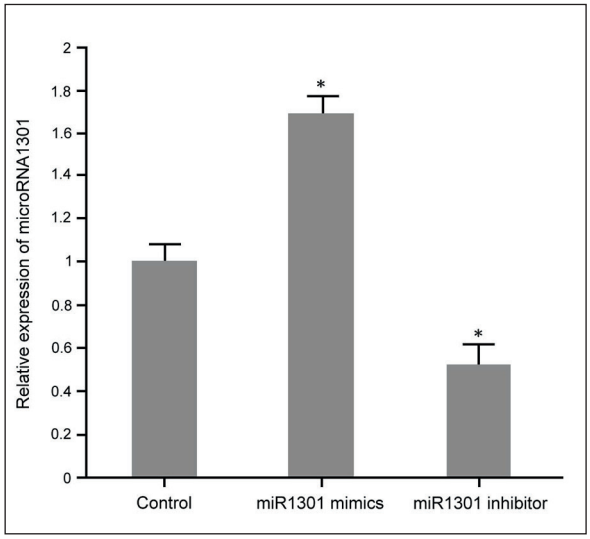

Figure 2. Effect of microRNA1301 on the expression of microRNA1301 in SKOV3/DDP cells. Compared with the control group, *$p < 0.05$.

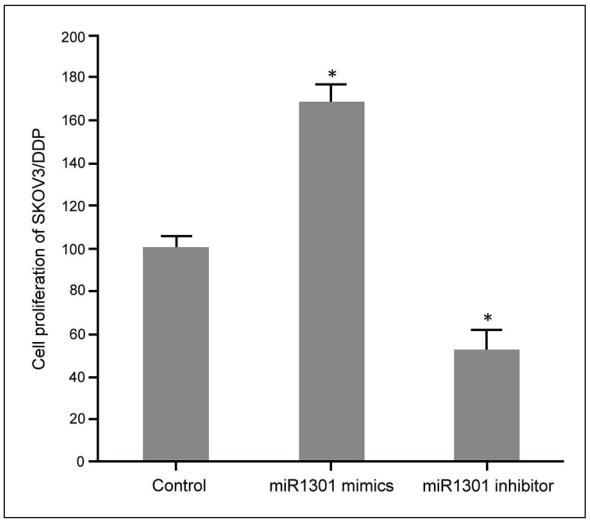

Figure 3. Effect of microRNA1301 on proliferation of SKOV3/DDP cells. Compared with the control group, *$p < 0.05$.

Effect of MicroRNA1301 on the Invasion Ability of SKOV3/DDP Cells

Transwell chamber analysis of the effect of microRNA1301 on the invasive ability of SKOV3/DDP cells showed that transfection of microRNA1301 mimics into SKOV3/DDP cells increased the expression of microRNA1301 in SKOV3/DDP cells, which further promoted the invasive ability of SKOV3/DDP cells. The difference was statistically significant ($p < 0.05$). Transfection of miR-1301 inhibitor into SKOV3/DDP cells decreased the expression of miR-1301 and inhibited SKOV3/DDP cell invasion. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 4)

Effect of MicroRNA1301 on the Expression of Autophagy Genes ATG5 and Beclin1 In SKOV3/DDP Cells

Real time PCR analysis of the effect of microRNA1301 on the expression of autophagy

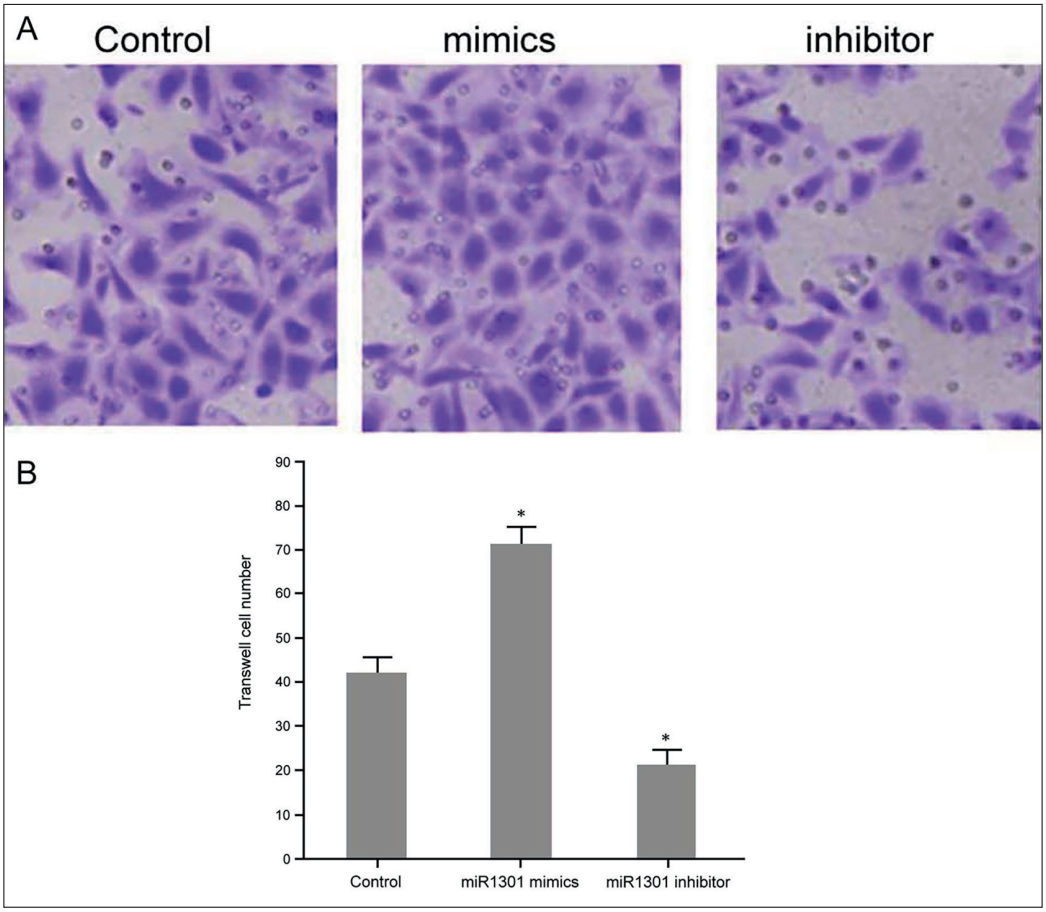

Figure 4. Effect of microRNA1301 on the invasion ability of SKOV3/DDP cells. **A**, Transwell chamber analysis of the effect of microRNA1301 on the invasive ability of SKOV3/DDP cells (×100); **B**, Statistical analysis of the effects of SKOV3/DDP cell invasion ability, compared with the control group, *$p < 0.05$.

genes ATG5 and Beclin1 in SKOV3/DDP cells showed that microRNA1301 mimics transfection into SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells, which inhibited the expression of ATG5 and Beclin1, compared with the control group, the difference was statistically significant ($p < 0.05$); miR-1301 inhibitor transfection into SKOV3/DDP cells resulted in decreased expression of miR-1301, which promoted ATG5 and Beclin1 expression. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 5).

Effect of MicroRNA1301 on the Expression EMT-Related Genes Snail and Slug in SKOV3/DDP Cells

Transfection of microRNA1301 mimics into SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells, which promoted the expression of EMT-related genes Snail and Slug. The difference was statistically significant ($p < 0.05$); miR-1301 inhibitor transfection into SKOV3/DDP cells resulted in decreased expression of miR-1301, which inhibited the expression of EMT-related genes Snail and Slug. Compared with the control group, the difference was statistically significant ($p < 0.05$; Figure 6).

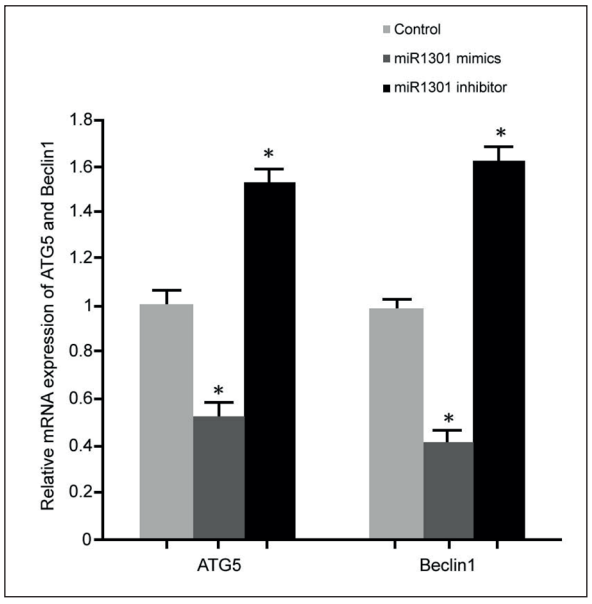

Figure 5. Effect of microRNA1301 on the expression of autophagy genes ATG5 and Beclin1 in SKOV3/DDP cells. Compared with the control group, *$p < 0.05$.

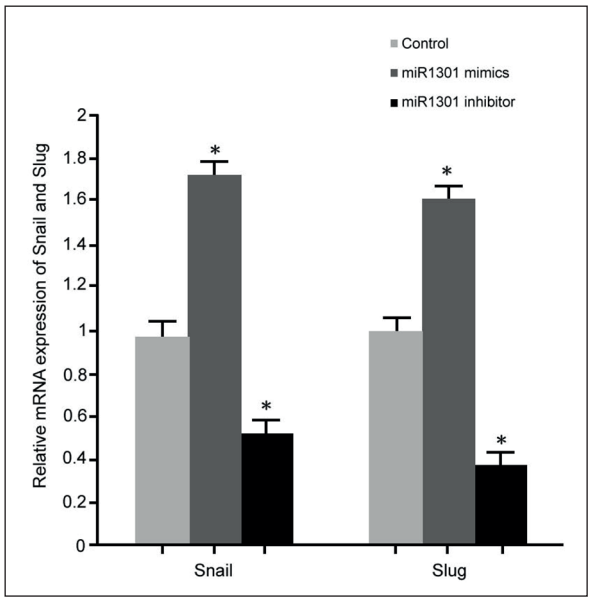

Figure 6. Effect of microRNA1301 on the expression of EMT-related genes Snail and Slug in SKOV3/DDP cells. Compared with the control group, *$p < 0.05$.

Effect of MicroRNA1301 on the Expression of NF-κB in SKOV3/DDP Cells

Transfection of microRNA1301 mimics into SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells and increased expression of NF-κB. Transfection of miR-1301 inhibitor into SKOV3/DDP cells resulted in decreased expression of miR-1301 and inhibited NF-κB expression (Figure 7).

Effect of MicroRNA1301 on the Expression of E-cadherin and N-cadherin in SKOV3/DDP Cells

Western blot analysis of the effect of microRNA1301 on the expression of E-cadherin and N-cadherin in SKOV3/DDP cells showed that transfection of microRNA1301 mimics into

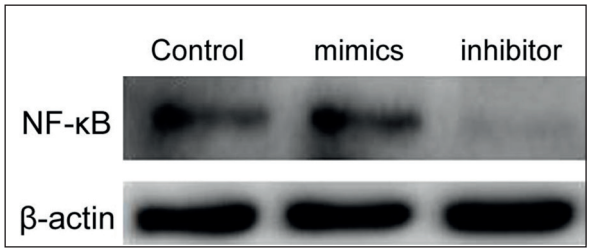

Figure 7. Effect of microRNA1301 on the expression of NF-κB in SKOV3/DDP cells.

SKOV3/DDP cells resulted in increased expression of microRNA1301 in SKOV3/DDP cells, decreased expression of E-cadherin, increased expression of N-cadherin, and transfection of miR-1301 inhibitor into SKOV3/DDP cells resulted in decreased miR-1301 expression, increased expression of E-cadherin, and decreased expression of N-cadherin (Figure 8).

Discussion

In recent years, chemotherapy resistance of ovarian cancer has attracted increasing clinical attention. Drug resistance can cause ovarian cancer treatment failure, and further development of tumor cell invasion and metastasis, resulting in easy recurrence and metastasis of ovarian cancer patients, ovarian cancer drug resistance induces tumor cell proliferation and increased invasion leads to poor prognosis and even an cause of death[17,18]. Research on tumor drug sensitivity-related miRNAs has received now growing attention. MiRNA expression is abnormal in the process of drug resistance of tumor cells, and the expression of related target proteins may also change, suggesting that miRNA and tumor resistance are closely related, and regulating the expression or function of miRNA may change the sensitivity to anti-tumor drugs[19]. MiRNAs inhibit E-cadherin expression by binding to the 3'-end of highly expressed EZH2, resulting in tumor cell epithelial mesenchymal transition (EMT) and oxaliplatin resistance[20]. MiRNA has a wide range of regulatory functions and plays an important role in physiological and pathological environments, and it can be involved in biological processes such as cell proliferation, differentiation, and apoptosis[21]. This research detected that the expression of microRNA1301 in ovarian cancer-resistant SKOV3/DDP cells was significantly increased, suggesting that microRNA1301 might be involved in the development of ovarian cancer resistance.

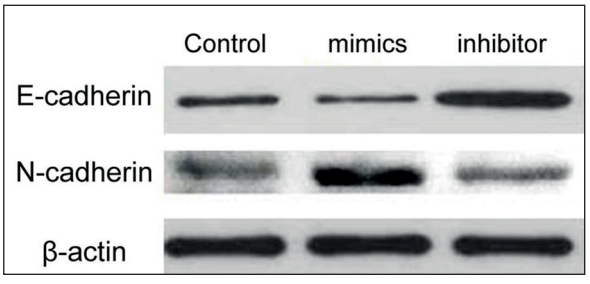

Figure 8. Effect of microRNA1301 on the expression of E-cadherin and N-cadherin in SKOV3/DDP cells.

This study further analyzed the role of microRNA1301 in the development and progression of drug resistance in ovarian cancer. In the physiological and pathological state of the body, autophagy is one of the self-degrading pathways of cells. Autophagosomes is the formation of vesicles in which the components to be degraded are encapsulated by lipid bilayer membranes. The lysosomal fusion vesicles then degrade the vesicles and their internal matter[22]. Autophagy can maintain cell homeostasis under normal physiological conditions, can fight tumors, and has stress resistance, which is an important mechanism of autonomic stabilization[8]. In the process of malignant tumor development, autophagy has a dual role in tumor suppression and tumor promotion, so the role of autophagy in tumors has gradually become a research hotspot[23]. In malignant tumors, inhibition or overexpression of autophagy key genes such as ATG5 and Beclin1 can play an important role in regulating the dynamics of autophagy, affecting the malignant proliferation or phenotype of NSCLC cell lines[24]. The process of EMT is closely related to the invasion and metastasis of tumors[25]. EMT involves multiple genes, and increased expression of Snail and Slug promotes EMT, and decreases the expression of cell adhesion factor E-cadherin, which not only blocks the adhesion between cells and adjacent cells, but also leads to invasion and metastasis of tumor cells to normal cells. In addition, increased expression of N-cadherin also causes epithelial cells to lose cell polarity, lose the epithelial phenotype with the connective basement membrane, and the migration and invasion of tumor cells[26]. This study indicated that microRNA 1301 expression was increased in SKOV3/DDP cells, and microRNA1301 mimics transfection into SKOV3/DDP up-regulated microRNA1301 expression, which further promoted cell proliferation and invasion, inhibited autophagy genes ATG5 and Beclin1 expression, and promoted EMT-related gene Snail and Slug expression, decreased expression of E-cadherin and increased expression of N-cadherin; microRNA130 inhibitors transfection into SKOV3/DDP cells can down-regulate microRNA1301 expression, promote autophagy genes ATG5 and Beclin1 expression, and inhibit EMT-related genes Snail and Slug expression, increase expression of E-cadherin and decrease expression of N-cadherin. The NF-κB signaling

pathway plays an important role as a key signaling pathway in autophagy and EMT, as well as in the process of cellular inflammatory response, tumor and immune response[27]. This work deteced that microRNA1301 mimics transfection into SKOV3/DDP up-regulated microRNA1301 expression, thereby promoting the increase of NF-κB expression. MicroRNA1301 inhibitor transfection into SKOV3/DDP cells down-regulated microRNA1301 expression, inhibited NF-κB expression, and then regulated human EMT and autophagy, thereby alleviating the development of SKOV3/DDP cells.

Conclusions

Briefly, targeting microRNA1301 can inhibit the proliferation of cisplatin-resistant cells and the development of EMT in human ovarian cancer by inhibiting NF-κB signaling pathway, therefore inhibiting the occurrence and development of drug-resistant ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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