

Original Article

Molecular mechanism of increased sensitivity of cisplatin to ovarian cancer by inhibition of microRNA-23a expression

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Abstract: Objective: The aim of this study is to investigate the sensitivity change and the preliminary mechanism of ovarian cancer cells on the resistant to chemotherapeutic drugs by inhibiting miR-23a expression. Methods: The ovarian cancer cell lines A2780 was administrated with antagomir-23a and platinum, and then the cell proliferation inhibition rate was determined by MTT assay. The cell cycle distribution was detected by flow cytometric analysis. The apoptotic morphological changes were analyzed by Hoechst33258 staining. The glycoprotein P-gp expression changes were detected by Western blot analysis. Results: The cell proliferation inhibition rate increased significantly after the administration of miR-23a and platinum ($P<0.01$). The middle concentration of drug efficacy IC_{50} in experimental group decreased by 83.76% compared with that in control group, which was 17.89 $\mu\text{mol/L}$ vs 110.18 $\mu\text{mol/L}$ ($P<0.01$). The cell lines A2780 were arrested in G0/G1 phase and apoptosis rate kept increasing ($P<0.05$). The cell nuclei stained by Hoechst33258 were obviously enhanced and demonstrated apoptosis morphology, such as condense, pyknosis. Compared with control group, the levels of P-gp protein expression in experimental group decreased along with the increase of the cisplatin concentration ($P<0.05$). Conclusion: The inhibition of miR-23a expression could significantly increase the sensitivity of cisplatin towards tumor cells, and it was probably because the negative regulatory factors of miR-23a target genes was released, and as a result, the expression of P-gp protein was inhibited.

Keywords: MicroRNA, cisplatin, ovarian cancer, drug resistance

Introduction

In recent years, more and more ovarian cancer patients produced resistance to platinum chemotherapy, and the comprehensive curative effect was reduced. As a result, the five-year survival rate of ovarian cancer patients significantly decreased [1]. It was an important research field in gynecologic oncology that the drug-resistance mechanism of ovarian cancer was discovered and the chemotherapy sensitivity of ovarian cancer was improved. The current discovery in the main drug-resistance mechanisms were the decrease of topoisomerases II- α of ovarian cancer, which reduced the effect of DNA double strand breaking by chemotherapeutic drugs [2]. Moreover, the levels of metallothionein (MTN) and multidrug resistance-associated protein (MRP) were up-regulated, which could protect the cell membrane from attacking by cisplatin, as one of the common clinical

drugs for the treatment of ovarian cancer [3]. Current researches focused on the abovementioned mechanisms of individual proteins, however, to clarify the drug-resistance mechanism of ovarian cancer, the precise mechanisms could not be illustrated depending on individual proteins.

MicroRNA was a large family of short, non-coding RNA, which had a wide regulation function at gene post-transcription level [4]. The expression of MiR-23a was obviously up-regulated in the chemotherapy-resistant ovarian cancer cell lines [5]. This study aimed to investigate the variation of drug-resistance and the preliminary mechanism in order to clarify the cisplatin drug-resistance mechanism of ovarian cancer, by reducing the level of MiR-23a in the cytoplasm of ovarian cancer cell lines A2780 with molecular biological methods.

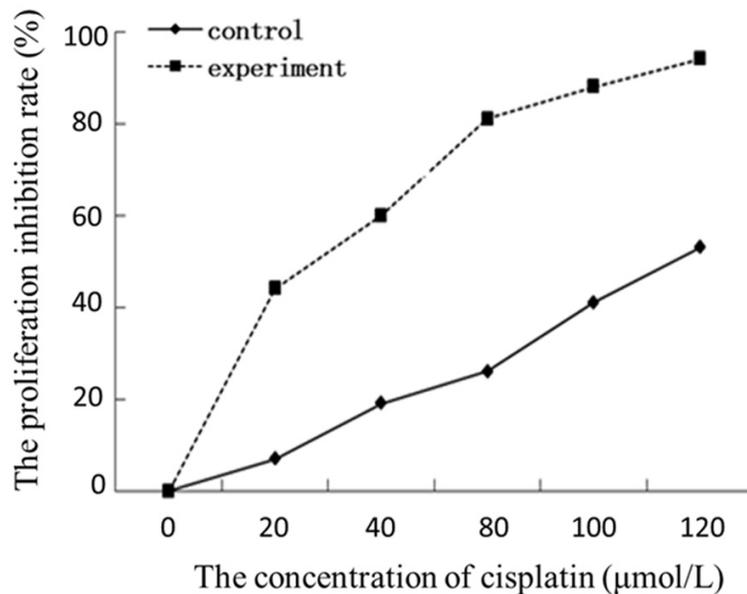


Figure 1. The effect of antagomir-23a on the CPIR of A2780 cells exposed to different concentrations of cisplatin.

Materials and methods

Materials and main instruments

The cisplatin drug-resistance ovarian cancer cell lines A2780 were donated by Dr. CHEN Guo-qin, obstetrics and gynecology department of the first affiliated hospital to Chongqin medical university. The antagomir-23a (miR-23a inhibitor) and Lipofectamine 2000 were purchased from Gima Biotech Company, Shanghai China. RPMI1640 were purchased from Sijiqin biotech company, Hangzhou China. Calf serum was purchased from Gibco Company, USA. MTT, total protein extraction kit, ECL, and Hoechst 33258 kit were purchased from Biyuntian Biotech Company, Jiangsu China. The murine anti-human P-gp monoclonal antibody, murine anti-human β-actin monoclonal antibody, and rabbit anti-mouse IgG secondary antibody were purchased from Baoshen Biotech Company, Dalian China. The flow cytometry was purchased from Becton Dickinson Company, USA.

Cell culture and experimental group

A2780 cells were cultured in culture solution with 10% fetal calf serum, in the incubator of 37°C and 5% CO₂. After the cells grew to pave completely, the cells would go through digestion, passage and inoculation. The cells were divided into control group and experimental

group. The cells of control group were incubated with cisplatin and the cells of experimental group were incubated with cisplatin and antagomir-23a.

MTT test

The cisplatin-resistant cell line A2780 cells paused at the logarithmic growth phase were inoculated into 96-well flat bottomed plates at a concentration of 3×10³ cells/ml. The cells of control group were incubated with cisplatin and the final concentrations were 0, 20, 40, 60, 80, 100, and 120 μmol/L, respectively. Each cell mass with final concentration were set into three holes. The cells in experimental group were added antagomir-23a with 5 pmol/hole, and Lipofectamine 2000 with 0.3 μL /hole in addition to cisplatin.

Each hole was routinely cultured for 48 h, and then added into 5 g/L×20 μL MTT solution to incubate for 4 h. The supernatant in each hole was abandoned, and each hole was added with 150 μL DMSO. After agitation for 5-10 min, the plates were sent to enzyme-labelled meter, and the A490 was detect at the 490 nm wave. The growth inhibition rate and IC₅₀ of each group were calculated with A490.

Flow cytometry analysis

The administration of drug was same to the MTT test. The cells were inoculated in 6-well flat bottomed plates in both control group and experimental group. After 48 h incubation, the cells were washed by PBS and reserved with 75% alcohol. Before the flow cytometry analysis, the cells were washed by PBS again and mixed with 180 μL 1% RNA RNA polymerase. When incubation at 37°C for 30 min, the cells were added into 800 μL PI in 4°C refrigerator for 30 min, and then was measured by FACS.

Apoptosis morphology detection

The drug treatments were the same to the FACS, and the Hoechst33258 staining was

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Table 1. The effect of antagomir-23a on the cell cycle distribution and apoptosis rate of A2780 cells exposed to cisplatin

Group	Cell cycle distribution and apoptosis rate (%)					
	Cisplatin inhibition		G0/G1phase	S phase	G2/M phase	apoptosis rate
Control group	0	0	46.15 ± 1.25	39.96 ± 1.76	13.89 ± 0.85	1.22 ± 0.14
	20	0	44.61 ± 2.36	40.42 ± 2.05	14.97 ± 0.91	2.10 ± 0.09
	40	0	48.97 ± 1.96	41.20 ± 1.33	9.83 ± 0.53	1.56 ± 0.27
	60	0	52.14 ± 1.52	38.58 ± 1.94	9.28 ± 0.61	3.69 ± 0.53
	80	0	55.53 ± 1.16	31.37 ± 2.14	13.10 ± 0.82	4.52 ± 0.46
	100	0	60.08 ± 2.37	27.51 ± 1.30	12.41 ± 0.13	7.04 ± 0.69
	120	0	64.91 ± 1.80	23.08 ± 2.22	12.01 ± 0.47	9.62 ± 0.85
Experimental group	0	0	49.02 ± 2.09	35.60 ± 3.15	15.38 ± 1.00	2.42 ± 0.24
	20	5	57.53 ± 2.41	33.61 ± 1.62 ^b	8.86 ± 0.72	3.19 ± 0.15
	40	5	63.18 ± 3.91 ^b	29.85 ± 1.51 ^b	6.97 ± 0.29	9.13 ± 0.61 ^b
	60	5	69.61 ± 1.92 ^b	23.10 ± 1.29 ^b	7.29 ± 0.43	13.66 ± 1.02 ^b
	80	5	76.39 ± 3.17 ^b	19.00 ± 1.10 ^b	4.61 ± 0.66	20.63 ± 1.16 ^b
	100	5	85.06 ± 2.85 ^b	7.15 ± 0.42 ^b	7.79 ± 0.34	34.13 ± 2.34 ^b
	120	5	91.49 ± 2.33 ^b	2.13 ± 0.19 ^b	6.38 ± 0.59	51.17 ± 3.91 ^b

Note: ^bP<0.05 mean the comparison of the same concentration between two groups. The concentration units of cisplatin and antagomir-23a were $\mu\text{mol/L}$ and pmol respectively.

used for apoptosis morphology detection. The cells were inoculated in 6-well flat bottomed plates in both control group and experimental group. After 48 h incubation, the cells were washed by PBS, fixed by 4% methanol and added with Hoechst33258 staining. After staining 3-5 min in dark room, the cells were observed under fluorescence microscope. The nuclei morphology of the apoptotic cells demonstrated the tight and hyperchromatic, or fragmental block structure.

Western blot detection

After 48 h incubation, the culture solutions of two groups' cells were abandoned. The protein quantification of two groups' cells was following the instructions of protein quantification kit. The two groups' cells were carried by Western blot detection. The first step was SDS-PAGE electrophoresis, transmembrane and seal. The system was added into primary antibody (murine anti-human P-gp monoclonal antibody, murine anti-human β -actin monoclonal antibody) at 4°C and for 12 h and then secondary antibody at 37°C and for 1 h. Finally, the system was sent to luminescence imaging system for imaging. The imaging was analyzed by Quantity One analysis software.

Statistical analysis

The data was analyzed by SPSS17.0 software. Continuous variables were reported as mean \pm standard deviation (SD), and compared using Student's t-test. Categorical variables were compared with Chi-square test. A *p*-value of less than 0.05 was considered as statistically significance.

Results

The effect of antagomir-23a on the cell proliferation inhibition rate (CPIR) of drug-resistance A2780 cell lines exposed to cisplatin

Compared with control group, the CPIR of experimental group was obviously higher (*P*<0.05) in MTT test. The effect was dose-dependent of cisplatin (**Figure 1**). The IC_{50} of control group was 110.18 $\mu\text{mol/L}$. The IC_{50} of experimental group was 17.89 $\mu\text{mol/L}$, less than control group by 83.76%.

The effect of antagomir-23a on the cell generation cycle and apoptosis rate of drug-resistance A2780 cell lines exposed to cisplatin

Compared with control group, the cells number of A2780 cell lines at phase S in experimental

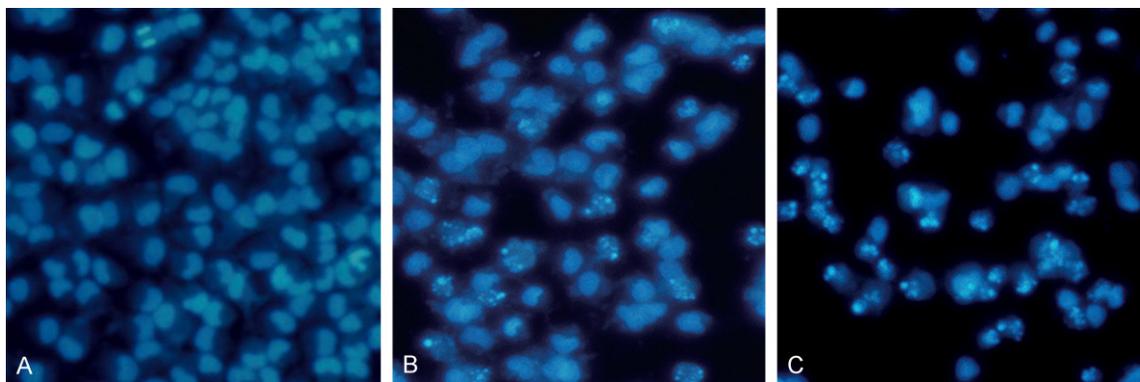
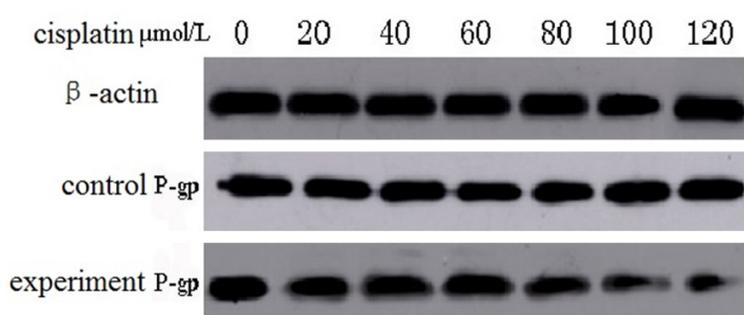


Figure 2. The effect of antagomir-23a on the morphology of apoptotic A2780 cells. A. Untreated. B. Treated with 60 μmol/L cisplatin. C. Treated with 60 μmol/L cisplatin and 5 pmol/L antagomir-23a.



The effect of antagomir-23a on P-gp protein expression to drug-resistance A2780 cell lines exposed to cisplatin

Figure 3. The effect of antagomir-23a on P-gp protein expression of A2780 cells exposed to different concentrations of cisplatin.

Compared with control group, the levels of P-gp protein expression in experimental group decreased along with the increase of the cisplatin concentration ($P < 0.05$). However, the levels of P-gp protein expression in control group was not obviously various

group was obviously less, but the cells number of A2780 cell lines at G0/G1 phase was obviously more. The A2780 cell lines were inhibited at phase G0/G1. The cell apoptosis rate of experimental group was obviously higher than that of control group (**Table 1**).

among all concentrations of cisplatin (**Figure 3; Table 2**).

The effect of antagomir-23a on the apoptosis morphology of drug-resistance A2780 cell lines exposed to cisplatin

Discussion

Compared with control group, the A2780 cell lines in experimental group were treated by cisplatin and antagomir-23a, and the cell nuclei stained by Hoechst33258 were obviously bright and demonstrated apoptosis morphology, such as condense, pyknosis. The cell apoptosis rate of mean concentration (60 μmol/L cisplatin) in control group was 4.23%, and the apoptosis rate of mean concentration (60 μmol/L cisplatin and 5 pmol/L antagomir-23a) in experimental group was 27.82% ($P < 0.05$) (**Figure 2**).

The ovarian cancer was the highest mortality of gynecological malignancy, and the incidence was significantly increasing recently. With the rapid improvement of the gynecological surgical procedures, the current therapy was mainly the comprehensive treatment of cytoreductive surgery and chemotherapy for intermediate and late-stage ovarian cancer. Because cytoreductive surgery could not eradicate the ovarian cancer cell *in vivo*, the chemotherapy was the last defense line and closely correlated with the patients' prognosis and 5-year survival rate [6]. In recent studies, the drug-resistance of chemotherapeutic agents of various kinds in tumor cells were confirmed, which led to poor therapeutic effect. Meanwhile, the adverse effect of chemotherapeutic agents could reduce the therapeutic effect of the comprehensive treatment. Therefore, the research of

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Table 2. The effect of antagomir-23a on the P-gp protein expression of A2780 cells exposed to different concentrations of cisplatin

group	Cisplatin concentration ($\mu\text{mol/L}$)					
	20	40	60	80	100	120
Control group	1.13 ± 0.02	0.95 ± 0.03	1.09 ± 0.02	0.87 ± 0.01	1.24 ± 0.04	1.21 ± 0.03
Experimental group	0.98 ± 0.01	0.94 ± 0.02	0.83 ± 0.03	0.51 ± 0.01^b	0.34 ± 0.01^b	0.23 ± 0.01^b

Note: ^b $P < 0.05$ mean the comparison of the same concentration between two groups. The concentration unit of cisplatin was $\mu\text{mol/L}$, and the antagomir-23a was pmol.

drug-resistance mechanisms of the ovarian cancer was crucial to improve the clinical therapeutic effect of ovarian cancer. This study proved that the inhibition of the cytoplasm expression of miR-23a of drug-resistance ovarian cancer A2780 cell lines could increase the sensitivity of ovarian cancer A2780 cell lines to the cisplatin.

MicroRNA (miRNA), a single-stranded RNA molecule with length of 20-22 nucleotides, was confirmed to exist widely in cytoplasm. miRNA does not have the protein encode function, but it inhibits the transcription of target genes protein by combining controlled target genes mRNA 3'-UTR sequence. Therefore, the regulatory function of the expression of target genes at post-transcriptional level was working. miR-23a was one of MicroRNA family and has widely gene regulation function [7]. This study findings proved that the sensitivity of ovarian cancer A2780 cell lines to the cisplatin obviously increased after the inhibition of drug-resistance ovarian cancer A2780 cell lines. The IC_{50} of experimental group was $17.89 \mu\text{mol/L}$, less than that of control group by 83.76%. This effect was cisplatin dose-dependent, which meant that the higher the concentration of cisplatin was, the higher was the cell proliferation inhibition rate (CPIR). The FACS analysis also pointed out that the ovarian cancer A2780 cell lines were inhibited at non-proliferative G0/G1 phase and cells at proliferative S phase obviously decreased by inhibition the expression of miR-23a and treatment with cisplatin. These changes could lead to increasing apoptosis rate. Meantime, the phenomenon of the increasing cell apoptosis was proved by morphology, which was the condensing and pyknosis of cell nucleus with bright staining. The morphology of cells treated by inhibited miR-23a and cisplatin changed significantly under the microscope, such as larger cell size, lower edge refractivity, different cell appearance liking "the rotten leaves" and conjugate division.

In order to investigate the molecular mechanism of the increase of A2780 cell sensitivity to the cisplatin by inhibiting the expression of miR-23a, this study measured the change of P-gp protein expression by Western blot detection. The presented study found that the expression of P-gp in experimental group obviously decreased. The human P-gp was encoded by tumor multi-drug resistance gene 1 (MDR1) mapped to No. 7 human chromosome. The molecular weight of human P-gp was 170 kD and it was formed by two same molecular structure subunits. Each subunit included six transmembrane domains and one ATP-binding site [8]. The P-gp protein was located at cytomembrane. It could play a role as drug pump to excrete the chemotherapeutic agents out of the cells when it combined with ATP. Therefore, it could reduce the accumulation of chemotherapeutic agents in order to avoid the cells injure. This resistance mechanism was also called as classic resistance mechanism [9]. The researcher reported that the miR-23a could combine the domain of RUNX3-3'UTR directly by the relative activity of luciferase analysis, so RUNX3-3'UTR gene was confirmed to be the regulatory target gene of miR-23a [10]. The Runx3 gene was one of anti-oncogenes and its tumor suppressor mechanism was correlated with TGF- β signal pathway. The TGF- β was a growth factor, which could inhibit the multiple development and physiological process [11, 12]. When the Runx3 protein was combined to transcriptional repressor Sin3A, the silent gene MDR1 was expressed. At last, the expression of P-gp protein decreased. Because of highly expressed level of miR-23a in drug-resistance tumor cell, the effect of carcinostasis to Runx3 gene was restricted.

Based on the presented results, we speculated the following conclusions: (1) The expression of miR-23a in drug-resistance ovarian cancer A2780 cell lines obviously increased; (2) The expression of Runx3 gene could be inhibited by

the combination of miR-23a and Runx3 3'UTR domain, which restricted the effect of Runx3 gene on the silence of MDR1 expression; (3) The expression of P-gp in drug-resistance tumor cell was obviously up-regulated, therefore the resistance mechanism was achieved by the classic resistance mechanism; (4) If the expression of miR-23a was inhibited, the regulatory effect decreased, and the expression level of Runx3 increased, and the silent effect of MDR1 expression by Runx3 improved. (5) The expression of P-gp decreased, so the classic resistance mechanism was also inhibited to various degrees, and then the sensitivity of cisplatin to drug-resistance increased. We demonstrate miR-23a had multiple-gene regulatory function, however the further studies should be performed to investigate the precise resistance mechanism.

Disclosure of conflict of interest

None.

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