EFFECT OF CIRC_0005075 ON DRUG-RESISTANT OVARIAN CANCER CELLS

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ABSTRACT

Objective: *The present study aims to explore the effect of circ_0005075 on drug-resistant ovarian cancer cells and its molecular mechanism.*

Methods: *A real-time fluorescent quantitative PCR (RT-qPCR) was used to detect the expression levels of circ_0005075 and miR-335 in ovarian cancer tissues, adjacent tissues, ovarian epithelial cells, ovarian cancer cell A2780 and taxolresistant cells A2780/Taxol; A2780/Taxol cells were divided into control (Con) group, si-NC group, si-circ_0005075 group, miR-NC group, miR-335 group, si-circ_0005075+anti-miR-NC group, si-circ_0005075+anti-miR-335 group. Tetramethylazolium salt colorimetric method (MTT) was used to detect cell viability; clone colony formation test was implemented to detect cell colony formation number; skin scratch test was carried out to detect cell scratch healing rate; Transwell was used to detect cell invasion number; Western blot method was used for detecting protein expression; dual-luciferase reporter experiment was conducted to detect the targeting relationship between circ_0005075 and circ_0005075.*

Results: *compared to adjacent tissues and ovarian epithelial cells, the expression level of circ_0005075 in ovarian cancer tissue, ovarian cancer cell A2780 and drug-resistant cells A2780/Taxol increased, and the expression level of miR-335 decreased (P<0.05). Due to interference with circ_0005075 or overexpression of miR-335, the expression level of miR-335 in drug-resistant cells A2780/Taxol increased; cell viability was decreased; cell colony formation decreased; cell scratch healing rate decreased; the number of invasive cells decreased; E-cadherin expression increased and the expression level of N-cadherin decreased (P<0.05). Based on targeted regulation of miR-335 by circ_0005075, the inhibition of miR-335 reversed the effect of interference with circ_0005075 on the proliferation, migration and invasion of drug-resistant ovarian cancer cells.*

Conclusions: *interference with circ_0005075 can inhibit the proliferation, colony formation, migration and invasion of drug-resistant ovarian cancer cells by up-regulating miR-335.*

Keywords: *circ_0005075, miR-335, ovarian cancer, drug resistance.*

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Introduction

Ovarian cancer is one of the global gynecological malignant tumors. Paclitaxel is one of the first-line chemotherapy drugs used for ovarian cancer. Chemotherapy resistance is the main cause of recurrence for ovarian cancer patients, seriously affecting the prognosis of patients and is also one of the difficult problems in the treatment of ovarian cancer[1, 2]. Studying the potential molecular mechanism of taxol resistance to improve the sensitivity of cancer cells is an important way to improve the therapeutic effect of ovarian cancer. circRNA is a kind of long non-coding RNA. Studies have shown that circRNA plays a key role in many cancers, including ovarian cancer[3]. It is reported that circ_0005075 is up-regulated in glioma tissue, this is associated with distant metastasis and poor prognosis of glioma patients; circ_0005075 stimulates the proliferation and metastasis of glioma

by down-regulating SIRT1[4]. Circ_0005075 is overexpressed in colorectal cancer tissues, promoting the proliferation and invasion of colorectal cancer cells[5]. Circ_0005075 promotes the progression of liver cancer by inhibiting miR-335[6].

However, the effect of circ_0005075 on ovarian cancer and its drug resistance is still unclear. It is reported that inhibiting the expression of miR-335 promotes the migration of ovarian cancer cells[7]. miR-335 has low expression in cisplatin-resistant A2780 cells; the overexpression of miR-335-5p reduces cell survival, enhances cisplatin-induced apoptosis and restores cisplatin sensitivity in ovarian cancer cells[8]. Silencingcirc_0035483 enhances the sensitivity of human renal cancer cells to gemcitabine by regulating miR-335[9]. However, it is not yet clear whether circ_0005075 affects the drug resistance of ovarian cancer by regulating miR-335.

Materials and methods

Sources of organizational materials

The cancer tissues and adjacent tissues of 27 ovarian cancer patients in our hospital were selected. All patients had complete clinicopathological data and had already signed an informed consent.

Cells and main reagents

Ovarian epithelial cells, ovarian cancer cellA2780 and human ovarian cancer cell taxol resistant strain A2780/Taxol were purchased from American ATCC; RPMI-1640 culture medium was purchased from Hyclone Company; Trizol reagent was purchased from Invitrogen Company; the fluorescence quantitative kit was purchased from Thermo Fisher Scientific Company. MTT kit, Giemsa staining solution and double luciferase reporter gene detection kit were purchased from Beijing Baiao Laibo Technology Co., Ltd.; Transwell chamber and Matrigel are purchased from BD Company in the United States; protein extraction kit was purchased from Beijing Kairuiji.

Transfection and grouping of cells

The A2780/Taxol cells were cultured in RPMI-1640 medium. The cells were transfected with si-NC, si-circ_0005075, miR-NC and miR-335, and were divided into si-NC group, si-circ_0005075 group, miR-NC group, and miR-335 group; conventional cultured A2780/Taxol cells were used as control group (Con); the A2780/Taxol cells were transfected with si-circ_0005075 and anti-miR-NC and anti-miR-335 respectively, and were divided into si-circ_0005075+anti-miR-NC group and si-circ_0005075+anti-miR-335 group.

Detection of expression levels of circ_0005075 and miR-335 real-time by fluorescent quantitative PCR (RT-qPCR)

Appropriate amounts of ovarian cancer tissue and adjacent tissues were added to Trizol to extract total RNA. The total RNA of ovarian epithelial cell, ovarian cancer cellA2780 and drug-resistant cell A2780/Taxol were also extracted by a Trizol reagent. Then, cDNA was synthesized, and PCR was performed according to the instructions of fluorescence quantitative kit. Finally, the relative expression was calculated by $2^{-\triangle\triangle Ct}$ method. Circ_0005075 and miR-335 took GAPDH and U6 as their internal reference respectively.

The upstream primer sequence of circ_0005075 was 5'-CAAATCTTGCGGCAACGC-3', and its downstream primer sequence was 5'-GCGGGAGT-GAAGATTCGA-3'; the upstream primer sequence of GAPDH was 5'-CCTTCCGTGTCCCCACT-3', and its downstream primer sequence was 5'-GCCTGCT-TCACCACCTTC-3'; the upstream primer sequence of miR-335 was 5'-ATCCAGTGCGTGTCGTG-3, and its downstream primer sequence was 5'-TGCT-TCAAGAGCAATAACGA-3'; the upstream primer sequence of U6 was 5'-GCTTCGGCAGCACATAT-ACTAAAAT-3', and its downstream primer sequence was5'-CGCTTCACGAATTTGCGTGTCAT-3''; the primers were synthesized by Shanghai Sangon Bio-tech Company.

Detection of cell proliferation activity by MTT

The cells were cultured for 48 h. 20 μL MTT solution was added to each well, and 150 μL DMSO was added after incubation for 4h. After oscillatory reaction for 10m, the absorbance (OD) was measured at 490 nm by a microplate reader.

Detection of the number of cell colony formation by clone colony formation test

1×10^4 cells /mL cell suspension was prepared from the cells in a logarithmic growth phase, and 100 cells per well were inoculated into the six-well plate. After 2 weeks, the culture was terminated when clones were visible to a naked eye. After being washed with PBS twice, the plate was fixed with methanol for 15 min, and stained with Giemsa for 30 min. Colonies with more than 50 cells were counted with low power optical microscope.

Detection of cell scratch healing rate by skin scratch test

After digestion, the cells were inoculated in a culture dish. The bottom of the dish was marked with a marker pen. The cells were cultured until they covered the bottom of the dish. Then, the gun head was used for scratching vertically in a horizontal line. The scratched cells were washed with PBS, and then continued to be cultured after changing the solution. After 0h and 48h of culture, photos were taken for observation. The healing rate of scratches was calculated with Image-Pro Plus6.0 software.

Detection of cell invasion number by transwell

100l diluted Matrigel was added to the upper chamber of Transwell chamber. After shaking evenly, the mixture was kept standing for solidification. Then, 100l cell suspension was added, and 500 μL serum-containing culture solution was added to the lower chamber. After being cultured at 37°C for 24h, the culture plate was taken out. After staining with 0.1% crystal violet for 30 min and being rinsed twice with PBS, the cells not having passed through the basement membrane on the upper layer were wiped off with cotton swabs. Five visual fields were randomly selected under an optical microscope (×200) to be photographed and counted.

Detection of protein expression by Western blot

According to the instructions of the kit, the total cell protein was extracted. 50μL protein sample was used for for SDS-PAGE, and then transferred to PVDF membrane. After sealing with 5% bovine serum albumin, primary antibody was added. After being kept overnight at 4°C, secondary antibody was added to be cultured at room temperature for 1h. Then, chemiluminescence reagent was used for to be developed. The gray level of protein band was analyzed by Image J software. The protein expression level was the ratio of target band to GAPDH band.

Dual-luciferase reporter experiment

Wild-type and mutant luciferase vectors circ_0005075 were constructed and co-transfected into A2780/Taxol cells with miR-NC and miR-335 respectively. luciferase activity was detected according to the instructions.

Statistical analysis

SPSS 20.0 software was used for statistical analysis, and the measurement data conforming to normal distribution was expressed by mean standard deviation (x±s). The comparison between the two groups was conducted by t-test, and the comparison between multiple groups was conducted by one-way ANOVA, with a significance level of $P<0.05$ indicating a statistically significant difference.

Results

Expression levels of circ_0005075 and miR-335

Compared to adjacent tissues, the expression level of circ_0005075 in ovarian cancer tissue increased, and the expression level of miR-335 decreased ($P<0.05$).

Compared to the ovarian epithelial cells, the expression level of circ_0005075 increased and the expression level of miR-335 decreased in the ovarian cancer cellA2780 and the drug-resistant cell A2780/Taxol ($P<0.05$) (Figure 1).

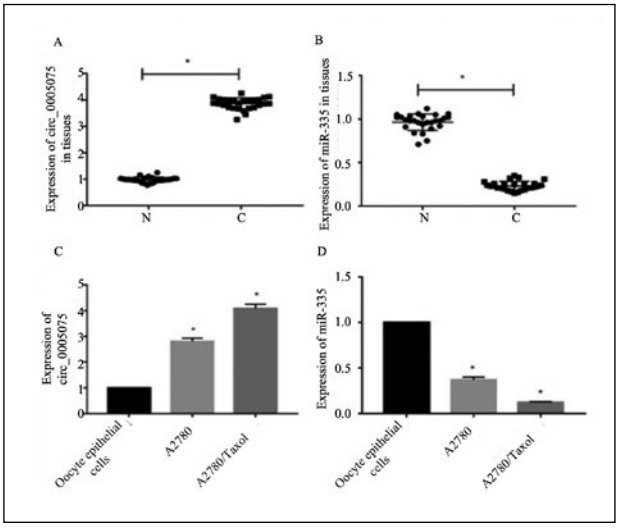

Figure 1: Detection of expression levels of circ_0005075 and miR-335.
N: adjacent tissues (27 cases) C: cancer tissues (27cases).

Effects of interference with circ_0005075 on proliferation of drug-resistant ovarian cancer cells

Compared to the Con group and si-NC group, the expression level of circ_0005075 in A2780/Taxol drug-resistant cells in si-circ_0005075 group decreased; the expression level of miR-335 increased; the cell activity decreased and the number of cell colonies decreased as well ($P<0.05$) (Table 1).

Effect of interference with circ_0005075 on migration and invasion of drug-resistant ovarian cancer cells

Compared to the control group and si-NC group, si-circ_0005075 group has decreased healing rate of cell scratches, has decreased a number of invasive

cells, has increased expression level of E-cadherin and has decreased expression level of N-cadherin (P<0.05) (Figure 2, Table 2).

Group	circ_0005075	miR-335	OD value	Number of colony formation(one)
Con	1.00±0.00	1.00±0.00	1.32±0.10	115.67±6.13
si-NC	0.99±0.01	1.02±0.03	1.33±0.12	116.33±6.18
si-circ_0005075	0.19±0.02*#	3.76±0.09*#	0.60±0.05*#	55.33±2.49*#
F	3888.600	2520.933	58.628	134.727
P	0.000	0.000	0.000	0.000

Table 1: Inhibition of cell viability and colony formation of drug-resistant ovarian cancer cells by interference with circ_0005075.
*Note: compared with Control group, *P<0.05; compared with si-NC group, #P<0.05.*

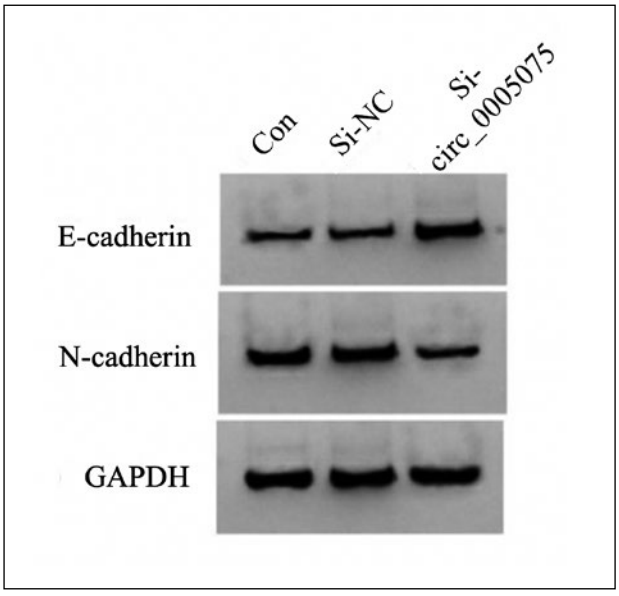

Figure 2: The effect of interference with circ_0005075 on the expression of E-cadherin and N-cadherin protein in drug-resistant ovarian cancer cells.

Group	Healing rate of scratches (%)	Number of invasive cells (one)	E-cadherin	N-cadherin
Con	65.30±4.30	144.00±6.16	0.13±0.01	0.70±0.07
si-NC	65.60±4.33	144.67±7.13	0.13±0.01	0.70±0.07
si-circ_0005075	35.19±1.88*#	67.67±3.40*#	0.59±0.05*#	0.23±0.02*#
F	67.378	175.734	235.111	64.971
P	0.000	0.000	0.000	0.000

Table 2: Inhibition of the migration and invasion of drug-resistant ovarian cancer cells by interference with circ_0005075.
*Note: compared with Control group, *P<0.05; compared with si-NC group, #P<0.05.*

Effect of miR-335 on migration and invasion of drug-resistant ovarian cancer cells

Compared to miR-NC group, miR-335 group has increased miR-335 expression level in drug-resistant cells A2780/Taxol, has decreased cell activity, has decreased number of cell colony formation, has decreased cell scratch healing rate, has decreased invasive cell number, has increased E-cadherin expression level and has decreased N-cadherin expression level (P<0.05) (Figure 3, Table 3).

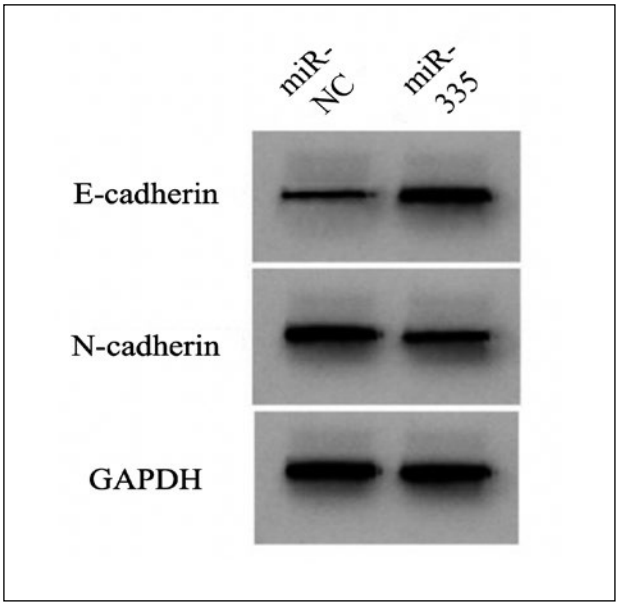

Figure 3: The effect of miR-335 on the expression of E-cadherin and N-cadherin protein in drug-resistant ovarian cancer cells.

Grouping	miR-335	OD value	Number of colony formation (one)	Healing rate of scratches (%)	Number of invasive cells (one)	E-cadherin	N-cadherin
miR-NC	1.00± 0.00	1.33± 0.12	116.33± 7.85	65.42± 4.39	144.66± 5.73	0.13± 0.02	0.69± 0.07
miR-335	3.38± 0.09*	0.71± 0.05*	64.33± 3.30*	38.65± 2.05*	82.33± 4.19*	0.52± 0.05*	0.27± 0.02*
t	45.803	8.261	105.77	9.570	15.209	12.544	9.992
P	0.000	0.001	0.000	0.001	0.000	0.000	0.001

Table 3: Inhibition of the proliferation, migration and invasion of drug-resistant ovarian cancer cells by miR-335.
*Note: compared with miR-NC group, *P<0.05.*

Targeting relationship between circ_0005075 and miR-335

Circular RNA Interactome online software predicted that circ_0005075 and miR-335 contained complementary sequences (Figure 4); the result of dual-luciferase reporter experiment showed that the luciferase activity of cells co-transfected with wt-circ_0005075 and miR-335 decreased, while the

luciferase activity of cells co-transfected with mut-circ_0005075 and miR-335 resulted in no significant change (Table 4).

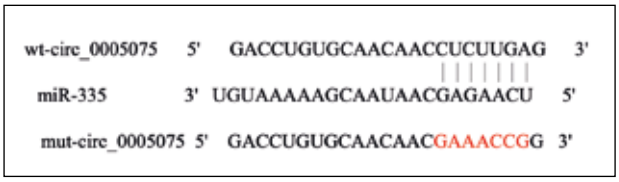

Figure 4: The complementary sequence of circ_0005075 and miR-335.

Group	wt-circ_0005075	mut-circ_0005075
miR-NC	0.93±0.06	0.98±0.08
miR-335	0.17±0.01*	0.93±0.05
t	21.641	0.918
P	0.000	0.411

Table 4: Double luciferase report experiment.
*Note: compared with miR-NC group, *P<0.05.*

Inhibition of miR-335 reverses the effect of interference with circ_0005075 on the proliferation, migration and invasion of drug-resistant ovarian cancer cells

Compared to si-circ_0005075+anti-miR-NC group, si-circ_0005075+anti-miR-335 group has decreased expression level of miR-335 in drug-resistant cells A2780/Taxol, has increased cell activity, has increased number of colony formation, has increased healing rate of cell scratches, has increased number of invasive cells, and has increased the number of e-cells (Figure 5, Table 5).

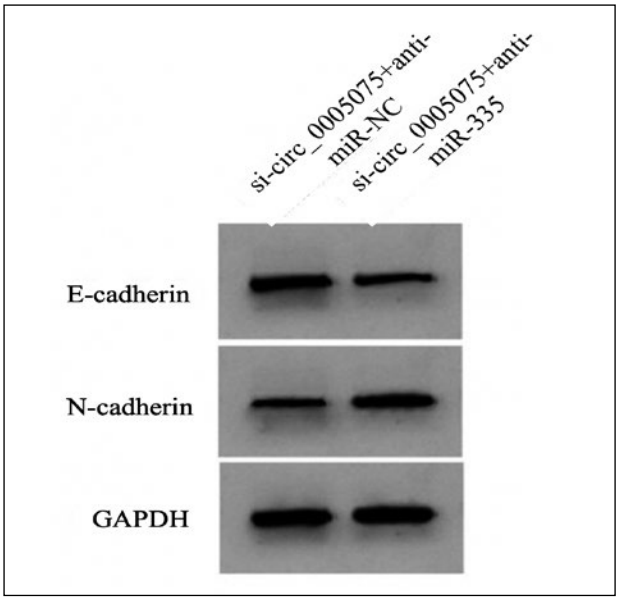

Figure 5: Inhibition of miR-335 reverses the effect of interference with circ_0005075 on the expression of E-cadherin and N-cadherin protein in drug-resistant ovarian cancer cells.

Group	miR-335	OD value	Number of colony formation (one)	Healing rate of scratches (%)	Number of invasive cells (one)	E-cadherin	N-cadherin
si-circ_0005075 +anti-miR-NC	3.75± 0.10	0.60± 0.05	55.33± 3.09	35.27± 1.63	68.00± 3.27	0.59± 0.06	0.23± 0.01
si-circ_0005075 +anti-miR-335	1.29± 0.07*	1.16± 0.06*	103.00± 5.10*	59.17± 2.15*	123.33± 6.13*	0.23± 0.01*	0.57± 0.05*
t	34.906	12.419	13.846	15.343	13.794	10.250	11.549
P	0.000	0.000	0.000	0.000	0.000	0.001	0.000

Table 5: Inhibition of miR-335 reverses the inhibitory effect of interference with circ_0005075 on the proliferation, migration and invasion of drug-resistant ovarian cancer cells.
*Note: compared with si-circ_0005075+anti-miR-NC group, *P<0.05.*

Discussion

Chemotherapy is an effective method to treat patients with advanced malignant tumors. However, tumor cells may become drug-resistant in the course of treatment, leading to a treatment failure. Studies have found that circRNA plays an important role in tumor drug resistance to chemotherapy, and studying its mechanism is expected to provide guidance for preventing and treating tumor drug resistance[10, 11]. The research reports that circCELSR1 (hsa_circ_0063809) regulates FOXR2 expression through miR-1252, affecting taxol resistance of ovarian cancer cells[12]. Circ_0005075, as a kind of circRNA, can enhance the proliferation, migration and invasion of colorectal cancer cells by activating Wnt /β-catenin pathway[13]. Circ_0005075 promotes the proliferation, migration and invasion of HCC cells by regulating miR-431[14]. The results of this experiment showed that the expression level of circ_0005075 increased in ovarian cancer tissue, ovarian cancer cellA2780 and drug-resistant cell A2780/Taxol.

After the interference with circ_0005075, the activity of drug-resistant cell A2780/Taxol decreased; the number of colony formation, the rate of scratch healing and the number of invasive cells decreased as well; the expression level of E-cadherin increased and the expression level of N-cadherin decreased. It is thus indicated that interference with circ_0005075 could inhibit the proliferation, migration and invasion of A2780/Taxol cells. It is reported that miR-335 regulates chemoradiation resistance of small lung cancer cells by targeting PARP-1[15]. The overexpression of miR-335 inhibited the viability and colony formation of GBC-SD and SGC-996 cells, making gallbladder cancer cells sensitive to

5-Fu[16]. Overexpression of miR-335 can increase the sensitivity of tumor cells to taxol, cisplatin and adriamycin, improving the chemotherapy effect[17]. The results of this experiment showed that the expression level of miR-335 in ovarian cancer tissue, ovarian cancer cellA2780 and drug-resistant cell A2780/Taxol decreased. The overexpression of miR-335 reduced the activity of drug-resistant cell A2780/Taxol, the number of colony formation, the rate of scratch healing, the number of invasive cells, the expression level of E-cadherin and N-cadherin, indicating that overexpression of miR-335 could inhibit the proliferation, migration and invasion of A2780/Taxol cells; moreover, this experiment found that the inhibition of miR-335 by a targeted regulation with circ_0005075 could reverse the effect of interference with circ_0005075 on proliferation, migration and invasion of drug-resistant ovarian cancer cells.

To sum up, interference with circ_0005075 inhibited the proliferation, colony formation, and migration of drug-resistant ovarian cancer cells by up-regulating miR-335, suggesting that interference with circ_0005075 was likely to improve the sensitivity of ovarian cancer cells to chemotherapeutic drugs.

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