Low expression of FXYD5 reverses the cisplatin resistance of epithelial ovarian cancer cells

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Low expression of FXYD5 reverses the cisplatin resistance of epithelial ovarian cancer cells

Running title: Effect of FXYD5 on cisplatin resistance in EOC

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Abstract

Objective: To investigate the effect of the downregulation of FXYD domain-containing ion transport regulator 5 (FXYD5) on the cisplatin resistance (CisR) of epithelial ovarian cancer (EOC) cells.

Methods: A2780-CisR and SKOV3-CisR cells were obtained through repeated administrations of different cisplatin concentrations, and the half-maximal inhibition concentration (IC50) was calculated by MTT assays. After transfection with FXYD5 siRNA-1 and FXYD5 siRNA-2, the IC50 values of the A2780-CisR and SKOV3-CisR cells were also detected by the MTT method. Cell proliferation, migration, invasion and apoptosis were evaluated through 5-ethynyl-2'-deoxyuridine (EdU) DNA synthesis, wound healing, Transwell invasion and Annexin-V-FITC/PI dual-staining assays, respectively. qRT-PCR and Western blotting were conducted to detect mRNA and protein expression.

Results: Compared with the sensitive parental cells, the A2780-CisR and SKOV3-CisR cells had increased IC50 and FXYD5 expression. FXYD5 siRNA reduced the IC50 value of cisplatin in the A2780-CisR and SKOV3-CisR cells and decreased the expression of ABCG2 (BCRP) and ABCB1 (MDR1). In addition, FXYD5 inhibition reduced the invasion and migration of the A2780-CisR and SKOV3-CisR cells, with upregulation of E-cadherin and downregulation of Snail and Vimentin. Both FXYD5 siRNA-1 and FXYD5 siRNA-2 inhibited the proliferation and promoted the apoptosis of the A2780-CisR and SKOV3-CisR cells with reduced Ki-67 and increased caspase-3.

Conclusion: FXYD5 downregulation may reduce the invasion, migration and EMT formation of EOC cells to increase their sensitivity to cisplatin chemotherapy by inhibiting cell proliferation and promoting cell apoptosis.
Key words: Epithelial ovarian cancer; FXYD5; cisplatin; Drug resistance
**Introduction**

Epithelial ovarian cancer (EOC) is the fifth major cause of cancer-related death in female patients, and it has the number one death rate of all gynecological cancers (Tassi et al., 2019). Most EOC patients are in the advanced/late stage at diagnosis due to occult onset, tumor heterogeneity and the high rate of metastasis (Qiu et al., 2019). Currently, a combination of cytoreductive surgery with platinum-taxane-based chemotherapy is the primary option for advanced-stage EOC patients, and 80% of them will suffer from recurrence (Cohen et al., 2019). Cisplatin has been widely used worldwide in cancer research for more than 30 years (Kucukkaya et al., 2019) since it has a good curative effect; however, its serious systemic side effects, such as nephrotoxicity, gastrointestinal reactions, neurotoxicity, ototoxicity, etc., often lead to strict restrictions on its dosage in clinical applications (Bentli et al., 2013; Van Acker et al., 2016). Moreover, many tumor cells show innate and/or acquired resistance to cisplatin, which further reduces the efficacy of platinum-anticancer drugs (Deng et al., 2020; Zhang et al., 2020). In particular, cisplatin resistance has always been a hot topic in cancer chemotherapy research.

FXYD domain-containing ion transport regulator 5 (FXYD5) is a type I membrane protein belonging to the seven-member FXYD family (FXYD1-7), and it contains a highly conserved F-X-Y-D motif in the transmembrane domain (Lubarski Gotliv, 2016). As shown by a previous study, FXYD5 is closely associated with the onset and progression of various diseases, and it can regulate epithelial morphology and epithelial cell expression through interaction with Na’-K’-ATPase (Wujak et al., 2016). FXYD5 is also known as “dysadherin” because its overexpression is correlated with the reduction of cell adhesion (Ino et al., 2002). The overexpression of FXYD5 to varying degrees is found in different types of tumor tissues, such as colorectal carcinoma (Aoki et al., 2003), thyroid carcinoma (Sato et al., 2003), and pancreatic ductal adenocarcinoma (Shimamura et al., 2003). Using a survival analysis of serous ovarian cancer (SOC) samples in TCGA database, Pichai Raman et al. identified FXYD5 as a potential metastatic marker of SOC, and its abnormal expression would result in a poor prognosis (Raman et al., 2015).
More importantly, Tassi RA et al. showed that FXYD5 upregulation was a predictor of shorter survival and platinum resistance in high-grade serous ovarian cancer patients (Tassi et al., 2019). Nevertheless, whether FXYD5 can reverse cisplatin resistance in EOC cells is unclear. Therefore, A2780 and SKOV3 EOC cell lines (Bai et al., 2015; Xia et al., 2015) were selected in this study to generate cisplatin-resistant (CisR) A278 and SKOV3 cells (A2780-CisR and SKOV3-CisR) using different concentrations of cisplatin. Then, two siRNA sequences (FXYD5 siRNA-1 and FXYD5 siRNA-2) were used for cell transfection to investigate the effect of FXYD5 downregulation on the drug resistance and biological characteristics of A2780-CisR and SKOV3-CisR cells.

Materials and methods

Cell culture

The human EOC cell lines A2780 and SKOV3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco’s modified Eagle’s medium (DMEM, Cat. #: D0819, Merck, Ltd., Beijing, China) used for cell culture was supplemented with 10% fetal bovine serum (FBS, Cat. #: F8687, Merck, Ltd., Beijing, China), and the conditions included 37°C and 5% CO₂. The solution was replenished once every 1-2 days. When cells covered 80% of the bottom area, they were subcultured, washed with PBS three times, digested with 1 ml of digestive solution containing 0.02% ethylenediaminetetraacetic acid (EDTA, Cat. #: 60-00-4, Merck, Ltd., Beijing, China) and 0.25% trypsin and placed at room temperature for 1 min. Digestion was terminated by 2 ml of culture medium containing 10% FBS. The cells were centrifuged at a rate of 1000 r/min for 5 min, the supernatant was discarded, and 5 ml of new DMEM culture medium containing 10% FBS was added to the centrifuge tube. The cell number was counted, and a single cell suspension was made, followed by cell culture at 37°C with 5% CO₂. Cells in the logarithmic growth phase were collected for later experiments.
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

A2780-CisR and SKOV3-CisR cells were obtained through repeated administrations with different increasing concentrations (0, 1, 2, 4, 6, 8, 16, 32, 64, 128 µM) of cisplatin (Cat. #: S1166, Selleck Chemicals, China), and the corresponding IC50 value was analyzed via MTT assays. A2780 and SKOV3 cells were collected at logarithmic phase, digested by trypsin and centrifuged to generate cell suspensions with a cell density of $5 \times 10^4$ cells/ml. The culture plate was placed into an incubator and cultured until the cell monolayer covered the bottom of the plate. Different concentrations of cisplatin (0, 1, 2, 4, 6, 8, 16, 32, 64, 128 µM) were added for 48 h of cell incubation at 37°C with 4.5% CO2. Next, MTT solution (5 mg/ml, Cat. #: B7777, ApexBio Technology, China) was added at 10 µl/well for another 4 h of incubation. Then, dimethyl sulfoxide (DMSO, Cat. #: D2650, Merck, Ltd., Beijing, China) was added at 150 µl/well for 10 min of shaking on the oscillator until all crystals were fully dissolved. The absorbance (OD value) was detected at OD 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Bioteck ELx800 Universal Microplate Reader, Bioteck Instruments, Inc., Vermont, USA). The half-maximal inhibitory concentration (IC50) was calculated, and the resistance index (RI) = IC50 (cisplatin-resistant cell)/IC50 (sensitive parental cell). The experiment was independently conducted three times.

Grouping and transfection of cells

A2780-CisR and SKOV3-CisR cells were divided into the control group (cells without any treatment), NC siRNA group (cells transfected with negative control siRNA: sense: 5'-UUCUCCGAACGUGACACGU-3'; antisense: 5'-ACGUGACACGUUCCGAGAATT-3'), FXYD5 siRNA-1 group (cells transfected with FXYD5 siRNA-1: sense: 5'-GAGUUCUGUGACUACGAU-3'; antisense: 5'-AUGAGUAGUACAGAUCUG-3'), and FXYD5 siRNA-2 group (cells transfected with FXYD5 siRNA-2: sense: 5'-CGAAUGGCCACUGCGAAU-3'; antisense: 5'-AUUCGCAGUGGCAUUUCGTT-3').
The NC siRNA (Cat. #: sc-37007), FXYD5 siRNA-1 (Cat. #: sc-45745) and FXYD5 siRNA-2 (Cat. #: sc-45746) were designed and purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). FXYD5 siRNAs were a pool of 3 target-specific 19-25 nt siRNAs designed to knock down FXYD5 expression. NC siRNA was used as a negative control for experiments using targeted siRNA transfection, which consisted of a scrambled sequence that would not lead to the specific degradation of any known cellular mRNA. Cell transfection was performed according to instructions using siRNA transfection reagent (Cat. #: sc-29528, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) in siRNA transfection medium (Cat. #: sc-36868, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The effects of different concentrations of cisplatin (0, 1, 2, 4, 6, 8, 16, 32, 64, 128 µM) on the IC50 and RI were measured using MTT assays.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of cells was extracted by TRIzol (Cat. #: 93289, Merck, Ltd., Beijing, China), and RNA concentration was quantified using an ultraviolet spectrophotometer. Then, the RNA was transcribed into cDNA using the PrimeScript® RT Regent Kit (Cat. #: RR037B, TaKaRa Bio, Inc., Shiga, Japan). PCR was carried out with the proper amount of cDNA as template. Primers were designed with Primer 5.0 software (Table 1) and synthesized by GenScript Biotech Corporation (Nanjing, China). qRT-PCR was performed according to the procedures of the PCR kit (kr011a1, Beijing Tiangen Biochemical Technology Co., Ltd., Beijing, China). The expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method (Rao et al., 2013) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal reference gene. The experiment was conducted three times independently.

Western blotting

The total protein was extracted, and the protein concentration was quantified using a bicinchoninic acid (BCA) kit (Cat. #: ab253410, Abcam, USA). The protein was separated via
polyacrylamide gel electrophoresis (SDS-PAGE, Cat. #: 89888, Thermo Fisher Scientific, USA) and transferred to a polyvinylidene fluoride (PVDF, Cat. #: 88585, Thermo Fisher Scientific, USA) membrane. The membrane was blocked in skim milk powder at room temperature and then washed with PBST buffer (0.05% v/v Tween-20 in PBS), followed by the addition of anti-FXYD5 at 0.2 µg/mL (Cat. #: PA5-52747, Thermo Fisher Scientific, USA), anti-BCRP at 1/1000 (Cat. #: ab207732, Abcam, USA), anti-MDR1 at 1/1000 (Cat. #: ab170904, Abcam, USA), anti-E-cadherin at 1/1000 (Cat. #: ab40772, Abcam, USA), anti-Snail at 1/1000 (Cat. #: ab216347, Abcam, USA), anti-Vimentin at 1/1000 (Cat. #: ab92547, Abcam, USA), and anti-β-actin-loading control at 1 µg/mL (Cat. #: ab8224, Abcam, USA) for 1 h of hybridization at room temperature. Then, the membrane was washed with PBST buffer for 3 min x 5 times, cultured for 1 h with goat anti-rabbit IgG H&L (HRP) at 1/2000 (Cat. #: ab97051, Abcam, USA) at room temperature for 1 h and washed again with PBST buffer (3 min x 5 times). The HRP substrate (Bio-Rad) was used to develop the target protein. Western blots were visualized using a Luminescent image analyzer (LAS-3000 Series, Fujifilm, Co., Tokyo, Japan), and protein expression was quantified by Multi gauge V3.0 software (Fujifilm, Co., Tokyo, Japan). The gray value ratio of the target protein to the loading control (β-actin) was taken as the relative content of the target protein. The experiment was repeated three times.

**EdU (5-ethyl-2'-deoxyuridine) DNA synthesis assay**

DNA synthesis was evaluated using Click-iT EdU Alexa Fluor 647 HCS Assay (Cat. #: C10640, Thermo Fisher Scientific, USA). The thymidine analog EdU was added to culture medium at a concentration of 10 µM for 24 h of incubation prior to culture termination. After fixation, the cells were permeabilized using 0.1% Triton-X, and an azide-modified Alexa Fluor 647 fluorescent probe was conjugated to the alkyne group of EdU that had been incorporated into nascent DNA using a copper-mediated “Click-iT” reaction. The reaction cocktail was replaced with rinse buffer, and counterstaining of cell nuclei was carried out with Hoechst stain (5 µg/mL) in Dulbecco’s...
phosphate buffered saline (DPBS) for 30 min. After rinsing, DPBS was added, and plates were stored at 4°C in the dark. Hoechst labeling (blue) indicated nuclei, EdU (red) indicated nascent DNA synthesis, and % EdU response normalized to the control group is shown. The experiment was repeated three times.

**Wound healing assay**

A marker pen was used to draw lines at the back of the 6-well plate with a ruler, with 0.5-1 cm of width between lines across the well. There were $5 \times 10^5$ cells in each well. The next day, the pipette tip was used to draw lines perpendicular to the lines at the back, and the gun head was vertical. PBS buffer was used for well washing (three times) to remove detached cells. Next, serum-free medium was added to culture cells at 37°C in a 5% CO₂ incubator. Cell samples were taken at 0 and 48 h for imaging under a light microscope (Olympus BX51; Tokyo, Japan). The percentage of wound closure (%) (Ker-Woon et al., 2015) was regarded as the ratio of wound area at 48 h to that at 0 h. The experiment was repeated three times.

**Transwell invasion assay**

The upper chamber of the Transwell (Cat. #: CLS3460, Merck, Ltd., Beijing, China) was covered with 50 mg/L Matrigel (Cat. #: E1270, Merck, Ltd., Beijing, China) at a 1:8 diluent before air drying at 4°C. The residual liquid in the culture plate was removed, and 50 µl of serum-free culture medium containing 10 g/L BSA was added into each well for 30 min of incubation at 37°C. Next, 300 µl of prewarmed serum-free medium was added to the upper chamber, which was exposed to air for 15-30 min at room temperature to rehydrate the Matrigel. The remaining culture medium was discarded. Before the preparation of the cell suspension, the cells were starved for 12 h, digested, and centrifuged. The culture medium was removed, and the plate was washed with PBS 1-2 times, followed by suspending cells in serum-free BSA-containing medium. When the cell density was adjusted to $5 \times 10^5$ cells/ml, the cell suspension was added to the Transwell cell
chamber, and 500 µl of FBS-containing medium was added to the lower chamber. After 48 h of
culture, the upper chamber was removed. Cells were fixed for 30 min with precooled 4% 
paraformaldehyde, stained for 1 min with 0.1% crystal violet, dehydrated with gradient ethanol, and
hyalinized with xylene. The polycarbonate film was cut from the substrate of the upper chamber
and placed on a glass slide for neutral resin sealing. At high magnification (× 400), 6 visual fields
were randomly selected for cell counting under a light microscope (Olympus BX51; Tokyo, Japan).
The experiment was repeated three times.

**Apoptosis detected by Annexin-V-FITC/PI**

Cell apoptosis was detected by using an Apoptosis Kit with Annexin V FITC and PI (Cat. #: 
V13242, Thermo Fisher Scientific, USA). Cells were centrifuged for 5 min, digested by trypsin
without EDTA, and centrifuged at 2000 rpm for another 5-10 min. Then, cells were resuspended in
precooled 1 × PBS (4°C), centrifuged at 2000 rpm for 5-10 min, washed, and suspended in 300 µl
of 1 × binding buffer. Next, 5 µl of Annexin V-FITC was added for 15 min of reaction at room
temperature in the dark. Approximately 5 min before sample loading, 5 µl of PI (propidium iodide)
was added. In addition, 200 µL of 1 × Binding Buffer was replenished before detection using
Attune NxT Flow Cytometry (Thermo Fisher Scientific, USA). The apoptosis rate of cells was
obtained after calculation with the formula: Apoptosis rate = early apoptosis rate (Annexin V+/PI−)
+ late apoptosis rate (Annexin V+/PI+) (Panattoni *et al.*, 2008). The experiment was repeated three
times.

**Statistical methods**

Statistical software SPSS 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism
6.0 (GraphPad Software, San Diego, CA, USA) were used for data analysis. The measurement data
are presented as the mean ± standard deviation. Comparisons between two groups were analyzed by
Student’s t-test, while one-way ANOVA or post hoc Tukey’s test was used for comparisons among
multiple groups. $P < 0.05$ indicated that the difference was statistically significant. GraphPad Prism 6 was used to calculate the IC50 value using the log (inhibitor) versus normalized response equation.

Results

FXYD5 expression in sensitive parent and cisplatin-resistant EOC cells

After EOC cells were exposed to different concentrations of cisplatin (0, 1, 2, 4, 6, 8, 16, 32, 64, 128 µM), an MTT assay was performed to detect the inhibitory effect of cisplatin on EOC cells. Compared with the A2780-par and SKOV3-par cells, the A2780-CisR and SKOV3-CisR cells had increased IC50 values with RIs of 4.83 and 5.05, respectively (Figure 1A-B and Table 2). The qRT-PCR results showed that the A2780-CisR and SKOV3-CisR cells had a 3.67-fold and a 4.02-fold upregulation of FXYD5 gene expression compared to the corresponding sensitive parent cells (both $P < 0.05$, Figure 1C). Additionally, a 2.86-fold and a 2.46-fold increase in FXYD5 protein expression was observed in the A2780-CisR and SKOV3-CisR cells compared with the A2780-par and SKOV3-par cells, respectively (both $P < 0.05$, Figure 1D-E).

Effect of FXYD5 siRNA on cisplatin resistance of the cisplatin-resistant EOC cells

To prevent potential off-target effects, we transfected A2780-CisR and SKOV3-CisR cells with two FXYD5 siRNA sequences (FXYD5 siRNA-1 and FXYD5 siRNA-2). The qRT-PCR analysis (Figure 2A) and Western blotting (Figure 2B-C) showed that FXYD5 siRNA-1 resulted in a 0.27-fold and 0.34-fold decrease in FXYD5 gene and protein expression in the A2780-CisR cells, and FXYD5 siRNA-2 led to a 0.28-fold and 0.33-fold reduction in the SKOV3-CisR cells ($P < 0.05$). Further analysis using MTT assays revealed that FXYD5 inhibition could effectively reverse the cisplatin resistance of the A2780-CisR and SKOV3-CisR cells, with significantly reduced IC50 values and RIs ($P < 0.05$, Figure 2D-E and Table 3).
**FXYD5 siRNA affects cisplatin resistance proteins in the A2780-CisR and SKOV3-CisR cells**

Using qRT-PCR, we found that compared with the NC siRNA, the FXYD5 siRNAs reduced the mRNA expression of *ABCG2* (FXYD5 siRNA-1: 0.40-fold in A2780-CisR cells and 0.55-fold in SKOV3-CisR cells; FXYD5 siRNA-2: 0.41-fold in A2780-CisR cells and 0.59-fold in SKOV3-CisR cells) and *ABCB1* (FXYD5 siRNA-1: 0.48-fold in A2780-CisR cells and 0.49-fold in SKOV3-CisR cells; FXYD5 siRNA-2: 0.46-fold in A2780-CisR cells and 0.47-fold in SKOV3-CisR cells) in the cisplatin-resistant EOC cells (*P* < 0.05, Figure 3A-B). In addition, Western blotting showed that the protein expression of BCRP decreased 0.31 times and 0.35 times in the A2780-CisR cells and 0.45 times and 0.48 times in the SKOV3-CisR cells, respectively, after transfection with FXYD5 siRNA-1 and FXYD5 siRNA-2 (all *P* < 0.05, Figure 3C-D). Moreover, the protein expression of MDR1 detected by Western blotting decreased in the FXYD5 siRNA-1 and FXYD5 siRNA-2 groups relative to the NC siRNA group (FXYD5 siRNA-1: 0.34-fold in A2780-CisR cells and 0.30-fold in SKOV3-CisR cells; FXYD5 siRNA-2: 0.32-fold in A2780-CisR cells and 0.33-fold in SKOV3-CisR cells, all *P* < 0.05, Figure 3C, E).

**Effects of FXYD5 siRNA on EMT, invasion and migration of A2780-CisR and SKOV3-CisR cells**

As shown in Figure 4 (wound healing assay) and Figure 5A (Transwell invasion assay), the inhibited expression of FXYD5 generated by FXYD5 siRNA-1 and FXYD5 siRNA-2 resulted in a decrease of at least 2-fold in the invasion and migration of the A2780-CisR and SKOV3-CisR cells (all *P* < 0.05). By detecting the expression of EMT-related genes using qRT-PCR, we found that *E-cadherin* expression increased by 3-fold and the expression of *Snail* and *Vimentin* decreased approximately 2-fold after transfection with FXYD5 siRNAs (*P* < 0.05, Figure 5B-C), and the protein expression examined by Western blotting demonstrated a similar trend, with upregulation of
E-cadherin and downregulation of Snail and Vimentin by at least 2-fold in FXYD5 siRNA-transfected A2780-CisR cells (Figure 5D) and SKOV3-CisR cells (Figure 5E).

**FXYD5 siRNA regulates the proliferation and apoptosis of A2780-CisR and SKOV3-CisR cells**

EdU results showed that FXYD5 siRNA-1 and FXYD5 siRNA-2 could significantly inhibit the proliferation of A2780-CisR (0.48-fold and 0.42-fold) and SKOV3-CisR cells (0.55-fold and 0.57-fold) compared with the control ($P < 0.05$, Figure 6A). In addition, Annexin V-FITC/PI staining revealed that both FXYD5 siRNA-1 and FXYD5 siRNA-2 could promote apoptosis by at least 4-fold in A2780-CisR cells and at least 3-fold in SKOV3-CisR cells (all $P < 0.05$, Figure 6B).

We detected the expression of proliferation- and apoptosis-related genes by qRT-PCR and found that Caspase-3 expression was more than 2 times higher in the A2780-CisR and SKOV3-CisR cells transfected with FXYD5 siRNAs than the control cells, along with a decrease of approximately 5 times in Ki-67 expression (all $P < 0.05$, Figure 6C-D).

**Discussion**

In this study, the expression of FXYD5 in cisplatin-resistant EOC cells was significantly increased, and we also found that the RI of A2780-CisR and SKOV3-CisR cells was decreased with the decreased expression of ABCG2 (BCRP) and ABCB1 (MDR1) after transfection with FXYD5 siRNAs. The ATP binding cassette (ABC) transmembrane transporter superfamily is usually upregulated in tumor cells, and these molecules can bind to anticancer drugs and utilize energy from ATP hydrolysis to pump drugs out of the cells, thereby inducing drug resistance of tumor cells (Naha *et al.*, 2020; Ren *et al.*, 2020). Similarly, FXYD5 knockdown was found to enhance cell sensitivity to various drugs, such as carboplatin, doxorubicin and fluorouracil, and reduce the expression of the ABC transporter gene ABCG2 in liver cancer stem cells (Jiang *et al.*, 2015). In addition, FXYD5 transfection resulted in the increased expression of ABCB1 in hepatocellular
carcinoma cells, as shown by Park JR et al. (Park et al., 2011). FXYD5, as an established modulatory subunit of Na⁺/K⁺-ATPase (NKA), was reported to be associated with several types of cancer and affect lethal outcomes by promoting metastasis, acting as an oncogene in many carcinomas (Lubarski Gotliv, 2016; Lubarski-Gotliv et al., 2017). The NKA enzyme can mediate the transport process, which has also been regarded as a potential mechanism affecting cisplatin uptake (Andrews et al., 1988; Hall et al., 2008). Cell lines that become resistant to cisplatin exhibit a lower expression of NKA than susceptible cell lines (Ahmed et al., 2009). In addition, cisplatin-sensitive cell lines have high expression of the Na and K ATPase α1 subunits after cisplatin treatment, which can be correlated with their sensitivity to the drug (Kishimoto et al., 2006). These results suggest that the possible effect of FXYD5 overexpression on cisplatin resistance in ECO cells may be associated with NKA. However, due to the limitations of time and funds, the specific mechanism has not been studied in depth in the current study and will be investigated in future research.

FXYD5 was also shown to confer to EOC cells with a mesenchymal phenotype and regulate cellular junctions, as well as mediate chemokine production and migratory properties (Tassi et al., 2019). EMT refers to the transformation from epithelial cells to stromal cells, during which cells are endowed with the potential for metastasis and invasion, so this process plays an essential role in the in situ invasion, distant metastasis and drug resistance of many different tumors (Shilnikova et al., 2018). Moreover, E-cadherin (an epithelial marker) and Snail and Vimentin (mesenchymal markers) are closely related to the induction of EMT (Li et al., 2016). In our study, inhibition of FXYD5 reduced EMT, with increased E-cadherin expression and decreased expression of Snail and Vimentin. As previously reported, abnormal O-glycosylation in carcinoma cells inhibited stable expression of FXYD5 expression and upregulated E-cadherin expression (Tsuiji et al., 2003). Many clinical studies have shown a negative correlation between FXYD5 expression and E-cadherin expression in tumor tissues (Sato et al., 2003; Nakanishi et al., 2004; Maehata et al., 2011; Subramaniam et al., 2011). Moreover, the downregulation of FXYD5 was found to inhibit cell
invasion and migration through the upregulation of E-cadherin expression (Ino et al., 2002; Besso et al., 2019), which was further supported by the results in our study. Notably, the cisplatin resistance of ovarian cancer was also found to be strongly influenced by EMT-related genes (Haslehurst et al., 2012). Thus, we hypothesized that FXYD5 inhibition regulates the expression of EMT-related genes to control the invasion and metastasis of tumors and eventually reverses the cisplatin resistance of EOC cells.

Furthermore, upregulated FXYD5 expression can potentiate the proliferation of vascular smooth muscle cells (VSMCs) (Duan, 2014). There was a significant correlation observed between FXYD5 overexpression and tumor size. For instance, FXYD5 expression was substantially different in papillary carcinomas of different tumor sizes (Batistatou et al., 2008). In addition, the degree of FXYD5 expression was related to the size of the primary tumor of the thyroid (Sato et al., 2003). In primary lung cancer and metastatic sites, upregulated expression of FXYD5 was significantly correlated with the size and metastatic status of tumors (Mitselou et al., 2010). Ki-67 is a nuclear antigen closely related to cell proliferation, and its abnormal expression can reflect the proliferation of tumor cells (Milione et al., 2020). Caspase-3 is a key apoptotic executor protein downstream of the apoptotic cascade, and its increased expression indicates increased cell apoptosis (Janicke et al., 1998). In our study, FXYD5 siRNA inhibited the proliferation and promoted the apoptosis of A2780-CisR and SKOV3-CisR cells, reduced Ki-67 expression, and increased caspase-3 expression, which supported the hypothesis that the FXYD5 siRNA-mediated enhancement of EOC sensitivity to cisplatin is associated with its ability to regulate tumor cell proliferation and apoptosis.

In summary, the expression of FXYD5 was apparently increased in cisplatin-resistant EOC cell lines. However, inhibition of FXYD5 expression can significantly reduce the IC50 value of cisplatin-resistant EOC cells to cisplatin and affect the biological characteristics of tumor cells.
Conflict of interest

The authors declare that they have no conflict of interest.
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### Table 1 Primers for qRT-PCR in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’-3’)</th>
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<tbody>
<tr>
<td>FXYD5</td>
<td>Forward: TCCCACTGATGACACCACGA</td>
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<tr>
<td></td>
<td>Reverse: AAAACCAGATGGCTTGAGGGT</td>
</tr>
<tr>
<td>ABCB1</td>
<td>Forward: CTATGCTGGATGTTTCCGGT</td>
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<tr>
<td></td>
<td>Reverse: GCTTTGGCATAGTCAGGAGC</td>
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<tr>
<td>ABCG2</td>
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<td></td>
<td>Reverse: TCTTCGCCAGTACATGGTG</td>
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<td>Snail</td>
<td>Forward: TCGGAAGCCTAACCTACAGCGA</td>
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<tr>
<td></td>
<td>Reverse: AGATGAGCATTGGCAGCGAG</td>
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<tr>
<td>Vimentin</td>
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<td></td>
<td>Reverse: CTGGATTTCCTCTTCTCTGTG</td>
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<td>Ki-67</td>
<td>Forward: GAGGTGTGCGAGAAAATCCAAA</td>
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<tr>
<td></td>
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<td>Caspase-3</td>
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<td>GAPDH</td>
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<tr>
<td></td>
<td>Reverse: ATGACAAGGTCGGCTCC</td>
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</tbody>
</table>
Legends

Figure 1 FXYD5 expression in sensitive parental and cisplatin-resistant EOC cells
Note: A-B: MTT assays detected the inhibitory effect of cisplatin (0, 1, 2, 4, 6, 8, 12, 16 µM) on the A2780-par and A2780-CisR cells (A), as well as the SKOV3-par and SKOV3-CisR cells (B) after 48 h treatment; C: The mRNA expression of FXYD5 detected by qRT-PCR; D-E: The protein expression of FXYD5 detected by Western blotting; The experiment was repeated three times.

Figure 2 FXYD5 siRNA reverses cisplatin resistance of A2780-CisR and SKOV3-CisR cells
Note: A: The mRNA expression of FXYD5 detected by qRT-PCR; B-C: The protein expression of FXYD5 tested by Western blotting; D-E: MTT assay was carried out to detect the cell inhibition of A2780-CisR cells (D) and SKOV3-CisR cells (E) after exposure to different concentrations of cisplatin (0, 1, 2, 4, 6, 8, 12, 16 µM) for 48 h; The experiment was repeated three times.

Figure 3 FXYD5 siRNA affects cisplatin-resistant proteins, including the mRNA expression of ABCG2 (A) and ABCB1 (B), as well as the protein expression of BCRP and MDR1 (C-E), in A2780-CisR and SKOV3-CisR cells
Note: A-B: The mRNA expression of ABCG2 (A) and ABCB1 (B) was detected by qRT-PCR; C-E: The protein expression of BCRP and MDR1 was determined by Western blotting. The experiment was repeated three times.

Figure 4 FXYD5 siRNA inhibits the migration of A2780-CisR (A) and SKOV3-CisR cells (B) assessed by wound healing assays.
Note: The experiment was repeated three times.

Figure 5 FXYD5 siRNA inhibits the invasion (A) and EMT (B-E) of A2780-CisR and SKOV3-CisR cells.
Note: A: FXYD5 siRNA inhibits the invasion of A2780-CisR and SKOV3-CisR cells evaluated by Transwell invasion assay; B-C: qRT-PCR detects the expression of EMT-related genes in A2780-CisR (B) and SKOV3-CisR cells (C), including *E-cadherin*, *Snail* and *Vimentin*; D-E: Western blotting detects the expression of EMT-related proteins in A2780-CisR (D) and SKOV3-CisR cells (E), including E-cadherin, Snail and Vimentin. The experiment was repeated three times.

**Figure 6** Effect of FXYD5 siRNA on the proliferation and apoptosis of A2780-CisR and SKOV3-CisR cells

Note: A: Cell proliferation was evaluated after EdU staining; Hoechst labeling (blue) indicates nuclei and EdU (red) indicates nascent DNA synthesis, and % EdU response normalized to Blank group is shown; B: Annexin V-FITC/PI dual staining detects apoptosis, and the apoptosis rate of each group was compared; C-D: qRT-PCR detects the expressions of *Ki-67* and *Caspase-3* in A2780-CisR cells (C) and SKOV3-CisR cells (D). The experiment was repeated three times.
A

Inhibition rate (%)

A2780-par
A2780-CisR

0.0 0.5 1.0 1.5 2.0 2.5
Log Cisplatin (µM)

B

Inhibition rate (%)

SKOV3-par
SKOV3-CisR

0.0 0.5 1.0 1.5 2.0 2.5
Log Cisplatin (µM)

C

Relative mRNA expression

FXYD5

A2780-par
A2780-CisR
SKOV3-par
SKOV3-CisR

0.0 1.0 2.0 3.0 4.0 5.0

D

Relative protein expression

FXYD5

A2780-par
A2780-CisR
SKOV3-par
SKOV3-CisR

β-actin

E

Relative protein expression

FXYD5

A2780-par
A2780-CisR
SKOV3-par
SKOV3-CisR

P < 0.05

P < 0.05

P < 0.05

P < 0.05
**HISTOLOGY AND HISTOPATHOLOGY**

**Figure A**

![Bar graph showing relative mRNA expression of FXYD5 in A2780-CisR and SKOV3-CisR cells.](image)

**Figure B**

![Western blots for FXYD5 and β-actin in A2780-CisR and SKOV3-CisR cells.](image)

**Figure C**

![Bar graph showing relative protein expression of FXYD5 in A2780-CisR and SKOV3-CisR cells.](image)

**Figure D**

![Graph showing inhibition rate of A2780-CisR cells treated with different doses of Cisplatin.](image)

**Figure E**

![Graph showing inhibition rate of SKOV3-CisR cells treated with different doses of Cisplatin.](image)
A2780-CisR cells

48 h

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

SKOV3-CisR cells

48 h

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

P < 0.05

P < 0.05
HISTOLOGY AND HISTOPATHOLOGY

**A2780-CisR cells**

- **Control**
- **NC siRNA**
- **FXYD5 siRNA-1**
- **FXYD5 siRNA-2**

**SKOV3-CisR cells**

- **Control**
- **NC siRNA**
- **FXYD5 siRNA-1**
- **FXYD5 siRNA-2**

**Graphs**

- Cell number/field
- Relative mRNA expression of E-cadherin, Snail, and Vimentin
- Relative protein expression of E-cadherin, Snail, Vimentin, and β-actin

**Results**

- **A2780-CisR cells**
  - Comparisons of cell number/field and mRNA expression levels between control and treated groups.
  - Statistical significance indicated by *P < 0.05*.

- **SKOV3-CisR cells**
  - Similar comparisons and statistical analysis as for A2780-CisR cells.
A2780-CisR cells

\[ P < 0.05 \]

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

SKOV3-CisR cells

\[ P < 0.05 \]

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

Ki-67
Caspase-3

\[ P < 0.05 \]

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2

\[ P < 0.05 \]

Control
NC siRNA
FXYD5 siRNA-1
FXYD5 siRNA-2