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Effect and mechanism of miR-217 on drug resistance, invasion and metastasis of ovarian cancer cells through a regulatory axis of CUL4B gene silencing/inhibited Wnt/β-catenin signaling pathway activation

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Abstract. – OBJECTIVE: To explore the effect and mechanism of miR-217 in cisplatin resistance, as well as invasion and metastasis of ovarian cancer by inhibiting the expression of Cullin 4B (CUL4B) and the activation of Wnt/β-catenin signaling pathway.

MATERIALS AND METHODS: Human ovarian cancer cell lines COC1 (cisplatin sensitive) and COC1/DDP (cisplatin resistant) were cultured and were used to construct the COC1 group and COC1/DDP group, respectively. COC1/DDP cells were divided into blank group, NC group, miR-217 mimic group, miR-217 mimic NC group, miR-217 inhibitor group, miR-217 inhibitor NC group, si-CUL4B group, si-CUL4B NC group, overexpressed (oe) oe-CUL4B group, oe-CUL4B NC group, and miR-217 mimic +oe-CUL4B group, with the identification of cell transfection simultaneously. Bioinformatics prediction and Dual-Luciferase reporter gene assay of the targeting effect of miR-217 on CUL4B were performed, followed by MTT assay for cell proliferation, associated with the measurement of median inhibitory concentration (IC50). Real-time quantitative PCR (qRT-PCR) detected the mRNA expression of miR-217 and CUL4B, and Western blotting for detecting CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin protein expression. The cell invasion of cells was detected by transwell assay, cell migration by cell scratch assay and cell apoptosis by flow cytometry.

RESULTS: Bioinformatics prediction and Dual-Luciferase reporter gene assay verified that CUL4B was a target gene of miR-217, and the latter could silence the expression of the former gene. Compared with COC1 group, the relative expression of miR-217 was significantly decreased, while CUL4B mRNA and protein expression, as well as Wnt1, Wnt3, Wnt3a and β-catenin protein expression were increased significantly, with evidently increased cell proliferation, IC50, invasion and migration, and decreased apoptosis rate in COC1/DDP group (all $p<0.05$). Compared with blank group and corresponding NC groups, miR-217 mimic group had increased expression of miR-217 and decreased expression of CUL4B; miR-217 inhibitor group showed decreased miR-217 expression while increased CUL4B expression; si-CUL4B group indicated no significant change of miR-217 expression but decreased CUL4B expression; oe-CUL4B group showed no difference in miR-217 expression but increased CUL4B expression; miR-217 mimic +oe-CUL4B had increased miR-217 expression and no change of CUL4B expression. Meanwhile, miR-217 mimic group and si-CUL4B group exhibited decreased Wnt/β-catenin, Wnt1, Wnt3, Wnt3a and β-catenin expression, decreased cell proliferation, IC50, invasion and migration, and increased apoptosis (all $p<0.05$). Furthermore, miR-217 inhibitor group and oe-CUL4B group revealed increased Wnt/β-catenin, Wnt1, Wnt3, Wnt3a and β-catenin expression, increased cell proliferation, IC50, invasion and migration, and decreased apoptosis (all $p<0.05$). NC group and miR-217 mimic +oe-CUL4B group showed no significant difference in the above indexes (all $p>0.05$). While compared with miR-217 mimic group, miR-217 mimic +oe-CUL4B group showed increased cell proliferation, IC50, invasion and migration, and decreased apoptosis (all $p<0.05$).

CONCLUSIONS: CUL4B gene is the target gene of miR-217. MiR-217 can silence the expression of this gene, and inhibit the activation of Wnt/β-catenin signaling pathway to enhance the cisplatin sensitivity and reverse drug resistance, inhibit cell invasion and migration, and promote cell apoptosis.

Key Words:

MiR-217, CUL4B gene, Wnt/β-catenin signaling pathway, Ovarian cancer, Cisplatin sensitivity.

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Introduction

Ovarian cancer is one of the three major malignant tumors of the female reproductive system, with a high mortality rate, ranking first in the incidence of gynecological malignancies[1]. Cytoreductive surgery is a common approach for the treatment of this type of cancer[2]. On this basis, platinum-based systemic chemotherapy is the standard treatment for ovarian cancer[3]. Chemotherapy failure is a critical factor affecting the comprehensive efficacy of ovarian cancer, and the main reason is the emergence of chemotherapy resistance[4]. So far, the mechanism of chemotherapy resistance in ovarian cancer is still unclear and remains to be elaborated. At the same time, tumor heterogeneity is one of the characteristics of malignant tumors, and there are significant individual differences in tumor occurrence, development, metastasis, drug-resistant response, healing and prognosis[5]. Gene heterogeneity is the major form of tumor heterogeneity, including gene variation, gene amplification and loss, gene expression profile changes and gene coding product change at the protein level, etc.[6]. Significantly, the deterioration of biological behavior and the presence of chemotherapy resistance in ovarian cancer are often accompanied by a series of abnormal gene expression and signaling pathway changes[7].

The occurrence and development of tumor involve many kinds of cell events and pathological processes. Wnt/β-catenin signaling pathway plays an important role in the regulation of embryonic development, cell proliferation and differentiation[8]. Among them, β-catenin is the gate molecule of the pathway and its quantity and state in cells have a decisive influence on the pathway[9]. There is no Wnt signal in the normal growth of the body, and almost no free β-catenin in the cytoplasm[10]. A small amount of β-catenin mainly exists in the cell membrane and participates in cell adhesion. In the presence of Wnt signals, namely, when the signaling pathway is activated, a large amount of β-catenin accumulates in the cytoplasm, inducing the abnormal accumulation of β-catenin into the nucleus and activating the downstream gene transcription, thus leading to abnormal proliferation, invasion and metastasis of tumor cells, promoting angiogenesis and chemotherapy resistance[11]. It has been detected that the abnormal activation of Wnt/β-catenin signaling pathway is closely related to a variety of human tumors[12,13].

Furthermore, prior studies have indicated that the ectopic expression of β-catenin in Wnt/β-catenin signaling pathway is closely related to the expression of Cullin 4B (CUL4B) gene in hepatocellular carcinoma and gastric cancer, etc.[14,15]. It is suggested that CUL4B gene may be the target gene of Wnt/β-catenin signaling pathway. CUL4B belongs to the Cullin gene family, which is an important part of ubiquitin ligase E3. It is abnormally overexpressed in a variety of tumors and plays an important role in tumor development[16]. In addition, miRNA is a category of small, noncoding, and single stranded RNAs containing 17-25 nucleotides that regulate gene expression at the post transcriptional level[17,18]. It was first discovered in 1993, and research shows that about 30% of human genes are regulated by miRNAs[19]. It is usually involved in tumor and other pathophysiological processes by regulating target genes and then regulating downstream target proteins. It plays an important role in cell differentiation, development, proliferation, programmed cell death (apoptosis), metabolism, etc.[20,21]. MiRNAs can be used as oncogenes or tumor suppressor genes to regulate the occurrence, development and metastasis of tumors[22,23]. MiR-217 has been reported to be associated with the proliferation, migration and invasion of ovarian cancer[24]; yet no relevant study has been reported from the aspects of its role in regulating the activation of Wnt/β-catenin signaling pathway in ovarian cancer.

It has been shown that Wnt/β-catenin signaling pathway is closely related to tumor cell growth, proliferation, survival metabolism, angiogenesis, invasion and metastasis, tumor drug resistance, tumor immune escape, etc. So far, there is rare study of miR-217 regulating CUL4B to modulate Wnt/β-catenin signaling pathway in ovarian cancer. The value of elaborating the regulatory axis of miR-217/CUL4B/Wnt/β-catenin signaling pathway may exert a practical value in improving the understanding and providing new insights for molecular therapy of ovarian cancer. Accordingly, our study would detect the expression of miR-217 and Wnt/β-catenin signaling pathway related proteins in ovarian cancer cells; meanwhile, CUL4B was identified as the direct target gene of miR-217 to further clarify the mechanism of miR-217 regulating Wnt/β-catenin signaling pathway. Besides, this study would explore how miR-217 regulated the biological behavior of ovarian cancer, such as proliferation, invasion, migration and apoptosis.

It is expected to provide a theoretical basis for understanding the pathogenesis, diagnosis and prognosis of ovarian cancer, and provide a new therapeutic target for this type of cancer, which has important clinical significance.

Materials and Methods

Cell Culture

Ovarian cancer cell line COC1 and resistant strain COC1/DDP were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Phosphate-buffered saline (PBS) was freshly prepared, sterilized with high pressure steam for 20 min, and stored at 4°C; 0.25% trypsin solution was prepared freshly, blown repeatedly until it was completely dissolved, filtered by 0.22 μm microporous filter and sterilized, and stored at 4°C. After the cells were resuscitated at 37°C, they were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) supplemented with 1% penicillin-streptomycin at 37°C in a routine culture medium with 95% saturated humidity and 5% CO_2. When growing to 80% fusion, cells were digested with Ethylenediamine tetraacetic acid (EDTA)-free trypsin (Sigma-Aldrich, St. Louis, MO, USA) and then subcultured, and the cells growing in logarithmic phase were taken in the subsequent experiment

Cell Grouping and Transfection

The cells were divided into COC1 group and COC1/DDP group, and cisplatin of 40 umol/l was given to each group. The COC1/DDP cells were transfected with Lipofectamine™2000 (purchased from Invitrogen, Carlsbad, CA, USA) according to different transfection protocols. In addition to a general control group of Blank group (without any treatment), the following 5 groups were established to explore biological characteristics of ovarian cancer cells and corresponding mechanism, which included miR-217 mimic group (miR-217 mimic sequence); miR-217 inhibitor group (miR-217 inhibitor sequence); si-CUL4B group (si-CUL4B sequence); oe-CUL4B group (oe-CUL4B sequence), with corresponding control groups of miR-217 mimic NC, miR-217 inhibitor NC, si-CUL4B NC and oe-CUL4B NC group, respectively; in addition, miR-217 mimic +oe-CUL4B group (co-transfection of miR-217 mimic sequence and si-CUL4B sequence) was established for reverse validation of the relationship between miR-217 and CUL4B in the development of ovarian cancer.

It shall be noted that the negative control of miR-217 mimic group, miR-217 inhibitor group, si-CUL4B group, oe-CUL4B group was set up, which were miR-217 mimic negative control (NC), miR-217 inhibitor NC si-CUL4B NC and oe-CUL4B NC group, respectively, with the aim to verify the transfection efficiency and to check the statistical difference of difference control groups for mechanism exploration. At the same time, the negative control of miR-217 mimic +oe-CUL4B group was set as miR-217 mimic NC +oe-CUL4B NC group and miR-217 mimic +oe-CUL4B NC group to check the successful transfection of miR-217 mimic +oe-CUL4B.

All the transfected sequences were synthesized by Shanghai Sagon. In terms of cell transfection and intervention, when the ovarian cancer cells were in good condition, the 6-well plate was taken, and the medium containing 10% FBS without penicillin-streptomycin was added into the plate well and cultured in 5% CO_2 incubator at 37°C for 24 h. When the cell fusion degree was 70-80%, Lipofectamine™2000 complex was prepared as follows: dilute 5 μL Lipofectamine 2000 with 250 μL Roswell Park Memorial Institute-1640 (RPMI-1640), mix gently, and incubate at room temperature for 5 min. Dilute target plasmids with 250 μL RPMI-1640, and then mix evenly, and incubate at room temperature for 5 min. After that, the above target plasmids were mixed with Lipofectamine 2000 as much as possible and incubated for 20 min. The prepared compound was added to 6-well plate, and wells of miR-217 mimic NC, miR-217 inhibitor NC, si-CUL4B NC and oe-CUL4B NC were set up, and the other one was blank group without special intervention, all of which were placed in the incubator of 5% CO_2 at 37°C. After 6 h, the old medium was replaced with RPMI-1640 containing 10% FBS and continued to culture. Finally, 48 h after transfection, the transfection efficiency was observed.

Bioinformatics Prediction of Target Gene and its Verification

Target Scan, miRanda, miRbase and other online tools were used to predict the target gene of miR-217, with the fragment sequence containing the action site obtained. For the extraction of DNA, genomic DNA was extracted from COC1/DDP cells and used as cloning template of miR-217 and CUL4B-3'-UTR fragments. CUL4B-3'-

UTR-WT and CUL4B-3'-UTR-MUT containing miR-217 conservative binding site were amplified, and the amplified products were used for gel electrophoresis. After recycling of the target gene, the target gene was connected with the vector to construct pGL3-PTEN-3-UTR and PTEN-3-UTR-MT vectors. The recombinant positive clones were selected from the transformed Escherichia coli competent cells. The cells were divided into four groups: group A: co-transfected NC sequence and wild-type CUL4B (Wt-CUL4B) 3'-UTR; group B: co-transfected miR-217 mimic sequence and Wt-CUL4B 3'-UTR; group C: co-transfected NC sequence and mutant-type CUL4B (Mut-CUL4B) 3'-UTR; and group D: co-transfected miR-217 mimic sequence and Mut-CUL4B 3'-UTR. After double enzyme digestion of the target gene, it was sent out for sequencing by Shanghai Sagon (Shanghai, China) following the insertion of positive fragment. The recombinant plasmid was transfected into the cells. The activity of the Dual-Luciferase was detected in the fluorescence detector. The experiment was repeated three times.

Real-Time Quantitative PCR (qRT-PCR) for Relative Expression Detection of MiR-217 and CUL4B

The cells of each group were collected 48 h after transfection. The total RNA was extracted according to the instructions of TRIzol (Invitrogen, Carlsbad, CA, USA). To be specific, the adherent cells were digested with 0.25% trypsin, and the single cells were collected into 1.5 ml Eppendorf (EP) tubes. The cells were washed with pre-cooled PBS for three times. Cells in each EP tube was added with 1 ml TRIzol solution to extract total RNA. The RNA solution was stored at -70°C. The absorption values of RNA at 260 m and 280 mm were measured by spectrophotometer (Thermo Fisher Scientific, San Jose, CA, USA), and the concentration and purity of RNA were calculated. The primers of miR-217, CUL4B, U6 and glyceraldehyde-3phosphate dehydrogenase (GADPH) were designed and synthesized by Shanghai Sagon (Shanghai, China) (Table I). The reverse transcription experiment system 20 μL, and the step was finished in accordance with the protocol of the EasyScript First-Strand cDNA Synthesis SuperMix (TransGenBiotech, Beijing, China). The reverse transcription reaction system included Reverse transcript, deoxy-ribonucleoside triphosphate (dNTP) Mixture, RNase inhibitor, stem-loop primers, U6 Reverse primer, RNA, Reverse Transcriptase and RNase Free dH_2O. The reaction conditions were reverse transcription reaction at 42°C for 10 min and then inactivation of reverse transcriptase at 95°C for 2 min. The prepared reaction solution was collected for fluorescent real-time quantitative PCR (qRT-PCR), which was completed according to the instructions of SYBR®Premix Ex Taq™ II Kit (TaKaRa, Dalian, China). The volume of the reaction system was 20 μL, which was composed of 10 μL SYBR Premix, 0.4 μL ROX Reference Dye (50×), 2 μL cDNA template, 0.8 μL each of the upstream and downstream primers and 6.0 μL sterilized distilled water. In the process, the reference gene of miR-217 was U6, and that of CUL4B was GAPDH. The reaction conditions were preset as pre-denaturation at 95°C for 10 s (1 cycle), followed by denaturation at 95°C for 5 s, annealing at 60°C for 34 s, and extension at 72°C for 30 s (45 cycles). $2^{-\Delta\Delta Ct}$ was used to calculate the multiple of expression of miR-217 or CUL4B compared with the control group. (The experiment was repeated 3 times.)

Western Blot for Protein Expression Detection of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin

Cells after 24 h of culture with the culture medium discarded were taken for this experiment. The cells were resuspended by adding 100 μl radio immunoprecipitation assay (RIPA) protein lysate (Thermo Fisher Scientific, San Jose, CA, USA) into each well and blown repeatedly for 3-5 min, followed by centrifugation at 12,000 rpm for 15 min at 4°C. The absorbed supernatant was the protein solution, which was

Table I. Sequence primers for qRT-PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-217	TACTCAACTCACTACTGCATCAGGA	TATGGTTGTTCTGCTCTCTGTGTC
CDH1	CCTGGAGTTTGTAGGGTTTGAT	GAGACGGTGGTAGAAGATTTGG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

stored at -70°C after repacking. Bicinchoninic acid (BCA) protein quantitative Kit (Beyotime, Wuhan, China) was used for protein quantitative analysis, with the addition of 200 μl BCA working solution into each well and incubation at 37°C for 30 min. An amount of 30 μg of the total protein was added in the sampling well of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel to compress the protein in the concentrated gel at a constant voltage of 60V. When the bromophenol blue band entered into the separation gel, the voltage was changed into 80V. After wet transfer to polyvinylidene difluoride (PVDF) membrane, electric transfer was performed at 250 mA for 2 h. The PVDF membrane washed by tris buffered saline (TBS; Shanghai Xin Yu Biotech Co., Ltd., Shanghai, China) for 3 times (5 min each time) was sealed with 5% skimmed milk powder (TBS preparation) for 2 h to reduce the non-specific antigen. According to the antibody specification, the corresponding antibody was diluted into the diluent of the first antibody, the first antibodies were CUL4B (rabbit monoclonal antibody, ab76470, 1:5,000), Wnt1 (rabbit polyclonal antibody, ab15251, 1:25), Wnt3 (rabbit monoclonal antibody, ab172612, 1:10,000), Wnt3a (rabbit monoclonal antibody, ab219412, 1:1,000), β-catenin (rabbit polyclonal antibody, ab27798, 1:500), and β-actin (rabbit polyclonal antibody, ab8227, 1:2500), all of which were from Abcam, Cambridge, UK. The sealed PVDF membrane was placed into a plastic dish, followed by the addition of the above antibodies respectively, and incubation at 4°C overnight. After Tris-Buffered Saline and Tween-20 (TBST) washing (15 min× 3 times) next day, the diluted horseradish peroxidase (HRP) labeled second anti-IgG (goat anti-rabbit, ab205718, 1:2000, Abcam, Cambridge, UK) was added in the same way and incubated at room temperature for 2 h, followed by TBST washing (15 min× 3 times). The enhanced chemiluminescence (ECL) labeling reagent A solution and B solution were mixed at the ratio of 1:1 in the dark to add into PVDF membrane. The gel was imaged by the Bio-Rad gel imager (Bio-Rad, Hercules, CA, USA), and SmartView Pro 2000 (UVCI-2100, Major Science, Saratoga, CA, USA) was used for photography. The optical density values of the gel images were displayed by Quantity One software. With β-actin protein as the internal reference, the ratio of optical density of target protein band and internal reference band was used for expressing the protein expression. The experimental data were repeated three times to calculate the mean and standard deviation.

3-(4,5)-dimethylthiahiazo-(-z-y1)-3,5-di-phenytetrazoliumromide (MTT) Assay for Cell Growth Detection

MTT colorimetry is a method to detect cell survival and growth. The detection principle is that the succinate dehydrogenase in the mitochondria of living cells can reduce the exogenous MTT to the water-insoluble blue purple crystal formazan and deposit in the cells, but the dead cells have no such function. Dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) can dissolve formazan in cells, and its wavelength value is measured at 570 nm by enzyme-linked immunometric meter, which can indirectly reflect the number of living cells. Within a certain range of cell number, the amount of MTT (Sigma-Aldrich, St. Louis, MO, USA) crystal formation is directly proportional to the cell number. The specific experimental steps were as follows: an amount of 2×10^3 cells/well of logarithmic growth were taken for inoculation on 96-well plate; after transfection, the cells were digested with trypsin, followed by centrifugation to make cell suspension, after which the cell density was adjusted to $5\text{-}10\times10^4$/ml. Cells at the density of 1×10^4 cells/well were inoculated into the 96-well plate, and the volume of each well was 200 μL. MTT method was used to detect cell growth after the termination of cell culture. The absorbance value (OD) of optical density was measured at 0, 1, 2, 3, and 4 d. An amount of 20 μL of MTT reagent (5.0 g/L) was added into each well, incubated in 37°C incubator for 48 h. With the discarding of the supernatant after incubation termination, 150 μL DMSO was added into each well for 10 min of shaking to dissolve the crystal completely. The OD value of each well was measured at 570 mm wavelength on the Microplate Reader. The cell proliferation curve was drawn with time as abscissa and OD as ordinate (repeat the experiment 3 times). The IC50 is defined as the drug concentration at which 50% of the cells survive in comparison with the control, which was calculated in this experiment to reflect the condition of cell growth.

Transwell Assay for Cell Invasion and Migration Detection

In cell invasion experiment, the extracellular matrix (ECM) glue was diluted with serum-free RPMI-1640 (Gibco, Rockville, MD, USA) in a

ratio of 1:1, and 30 μL was added on the transwell polycarbonate membrane each chamber to cover the ECM gel. The membrane was placed into an incubator at 37°C for 15 min to make it solidify. After transfection for 24 h, the cells were digested with 2.5% trypsin and suspended in a serum-free DMEM medium to adjust the cell density to 1×10^6/mL. An amount of 200 μL of single cell suspension was added to the upper chamber, and 600 μL of RPMI-1640 medium containing 10% FBS was added to the lower chamber. The medium was placed in an incubator for routine culture at 37°C for 24 h. Three parallel wells were set in each group, and the experiment was repeated three times. After incubation, the chamber was taken out, with the matrix glue and cells wiped gently on the chamber, followed by staining with 0.19% crystal violet for 5min, and rinsing off the excess dye with clear water. Under ×200 microscope, the cells passing through the membrane were counted and photographed, and 5 visual fields (up, middle, down, left and right) were randomly selected and their average values were taken.

Cell Scratch Assay for Cell Migration Detection

In the cell migration experiment, the cells in good condition were digested and seeded in the 6-well plate. RPMI-1640 medium containing 10% FBS was added and cultured in the cell incubator for 24 h of cell culture to form monolayer cells. After that, the monolayer cells were scratched with the tip of a 10 μL pipette, after which the cells were washed with PBS for 3 times, followed by the performance of the intervention culture according to the experimental design. In the final step, the cell migration was observed at the same position under the inverted microscope every other period of time, and the cell growth change following the scratch assay was photographed at 0 h and 48 h, respectively.

Annexin V-FITC/Propidium Iodide (PI) Dual-Staining Assay for Cell Apoptosis Detection

In this experiment, cell apoptosis was detected by flow cytometry 48 h after transfection. Cells in each group were digested with trypsin without EDTA, and single cell suspension was prepared. Cells were washed with PBS twice to collect 1×10^5 cells and added with 500 μL binding buffer for the suspension of cells. Then, 10 μL Annexin V-FITC (Sigma-Aldrich, St. Louis, MO, USA) was added to mix well, followed by the addition and mixing of 5 μL PI (Sigma-Aldrich, St. Louis, MO, USA). After reaction in the dark at room temperature for 15 min, cell apoptosis detection was performed, and the experiment was repeated three times.

Statistical Analysis

SPSS 21.0 (SPSS, IBM, Armonk, NY, USA) software package was used to analyze the data and calculate the mean value and standard deviation. All experiments were repeated three times. The *t*-test is used for inter-group data analysis; meanwhile, in the case of homogeneous variances, one-way analysis of variance and LSD post-hoc test were used for multiple data analysis; while for the uneven variance, Welch's test and Dunnett's T3 test were used to compare the differences among groups. The difference was statistically significant when $p < 0.05$.

Results

CUL4B Verified to be the Target Gene of MiR-217

Using bioinformatics prediction software (microrna.org), it was found that miR-217 had a common binding site with 3'-UTR of CUL4B gene (Figure 1A). Dual-Luciferase reporter assay indicated that compared with NC and Wt-CUL4B plasmid co-transfection, the fluorescence of miR-

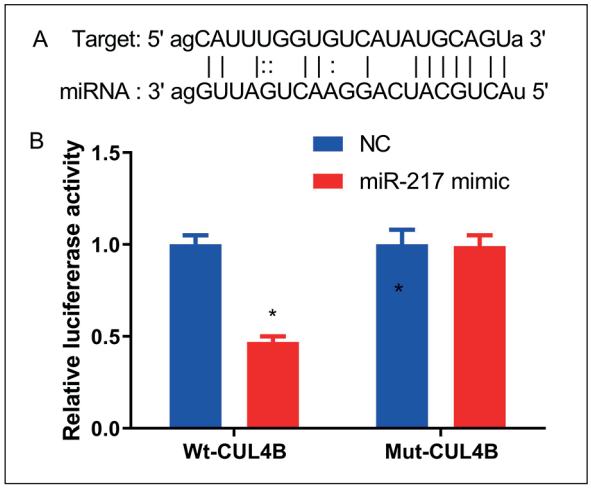

Figure 1. Target relationship verification between miR-217 and CUL4B. **A**, Bioinformatics prediction of the 3'-UTR binding sites of miR-217 and CUL4B; **B**, Determination of 3'-UTR luciferase activity of CUL4B (*compared with co-transfection group of miR-217 NC and miR-217 Wt-CUL4B, $p<0.05$). Wt: wide type; Mut: mutant type.

217 mimic and Wt-CUL4B plasmid co-transfection group decreased significantly ($p<0.05$). There was no significant difference in fluorescence when Mut-CUL4B was co-transfected with NC and miR-217 mimic, respectively ($p>0.05$). It was preliminarily showed that miR-217 was directly bound to 3'-UTR of CUL4B gene to suppress the expression of this gene.

Identification of Plasmid Transfection

According to the results of qRT-PCR (Figure 2), miR-217 was highly expressed in miR-217 mimic group, while lowly expressed in miR-217 inhibitor group than that in miR-217 mimic NC group and miR-217 inhibitor NC group, respectively (all $p<0.05$); meanwhile, CUL4B expression was down-regulated in si-CUL4B group but up-regulated in overexpressed (oe) oe-CUL4B group when compared to corresponding control of si-CUL4B NC group and oe-CUL4B NC group, respectively (all $p<0.05$). Besides, compared with miR-217 mimic NC+oe-CUL4B NC group, miR-217 mimic+oe-CUL4B NC group showed increased expression of miR-217 but decreased expression of CUL4B; while miR-217 mimic+oe-CUL4B group had increased miR-217 but no expression change in CUL4B. The above results suggested a successful transfection in each group according to different experimental grouping protocols.

Relative Expression of MiR-217 and CUL4B in Each Group after Cell Transfection

As shown in Figure 3, the expressions of miR-217 in COC1/DDP group were significantly lower while that of CUL4B was significantly higher than that in COC1 group (all $p<0.05$). Furthermore, compared with the blank group, there was no statistical difference in terms of the expression of miR-217 and CUL4B in miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group and oe-CUL4B NC group (all $p>0.05$). Compared with miR-217 mimic NC group, the expression of miR-217 was up-regulated and CUL4B was down-regulated in miR-217 mimic group, however, that of miR-217 was down-regulated and CUL4B was up-regulated in miR-217 inhibitor group in relative to miR-217 inhibitor NC group. Furthermore, there was no significant difference in the relative expression of miR-217 in si-CUL4B group and oe-CUL4B group when compared with si-CUL4B NC group and oe-CUL4B NC group, respectively, but with decreased expression of CUL4B in si-CUL4B group and increased expression in oe-CUL4B group. Meanwhile, miR-217 mimic +oe-CUL4B group showed increase in the expression of miR-217 compared with the blank group, but no significant change in CUL4B expression (all $p<0.05$). Besides, compared with miR-217 mimic group,

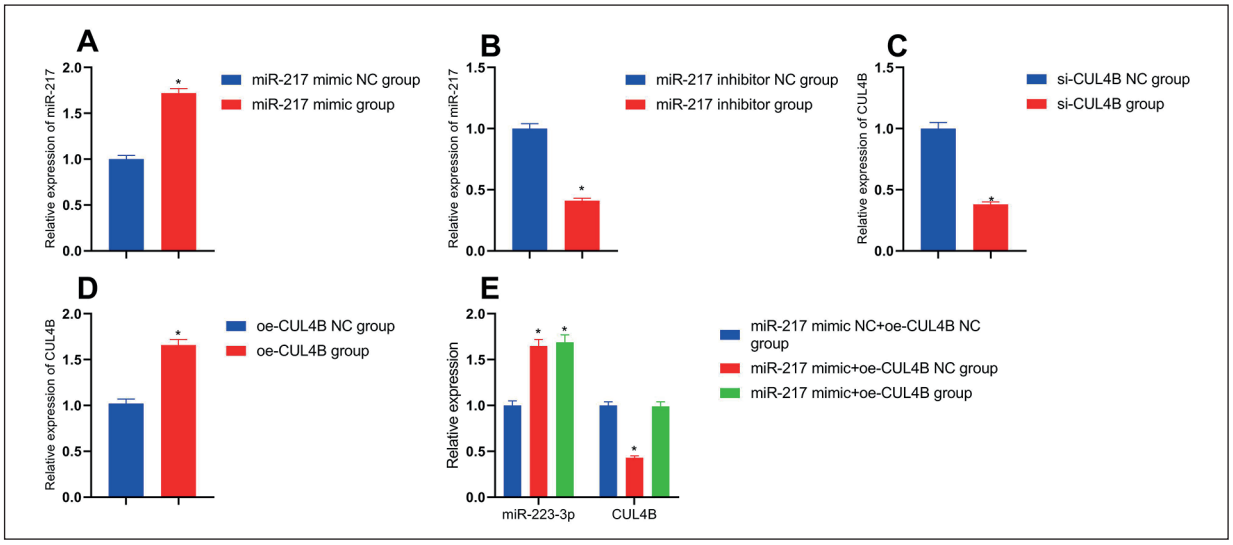

Figure 2. Cell plasmid transfection identification in each group with qRT-PCR. **A-B**, Expression of miR-217 after miR-217 mimic and inhibitor transfection, *compared with miR-217 mimic NC group and miR-217 inhibitor NC group, respectively, $p<0.05$; **C-D**, Expression of CUL4B after si-CUL4B and oe-CUL4B transfection, *compared with si-CUL4B NC group and oe-CUL4B NC group, respectively, $p<0.05$; **E**, Expression of miR-217 and CUL4B among miR-217 mimic NC+oe-CUL4B NC group, miR-217 mimic+oe-CUL4B NC group and miR-217 mimic+oe-CUL4B group, *compared with miR-217 inhibitor NC+oe-CUL4B NC group; $p<0.05$.

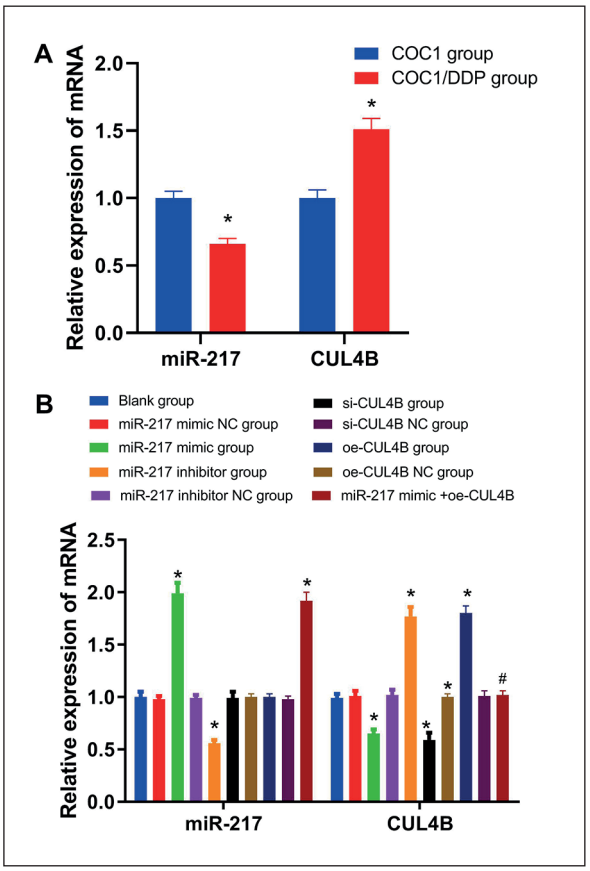

Figure 3. mRNA expression level of miR-217 and CUL4B in ovarian cancer cells after transfection in each group. **A**, The expressions of miR-217 and CUL4B in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; **B**, The expressions of miR-217 and CUL4B in different transfection groups, *compared with blank group and corresponding NC groups, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$.

miR-217 mimic +oe-CUL4B group showed no change of miR-217 expression but increased expression of CUL4B (all $p<0.05$). These results proved that CUL4B was a target gene of miR-217, and miR-217 could downregulate the expression of CUL4B.

Protein Expression of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin in Each Group After Cell Transfection

First, as for those protein expressions between COC1 group and COC1/DDP group, there were increased trends in the protein expressions of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin protein expression in the latter group than that in the former group (all $p<0.05$).

The results showed that compared with the Blank group, there was no statistical difference in terms of the protein expression of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin in miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group, oe-CUL4B NC group and miR-217 mimic +oe-CUL4B group (all $p>0.05$). Furthermore, the CUL4B protein expression was down-regulated in miR-217 mimic group and si-CUL4B group, but up-regulated in miR-217 inhibitor group and oe-CUL4B group in relative to corresponding control groups (all $p<0.05$); while there was no significant difference in CUL4B protein expression in miR-217 mimic +oe-CUL4B group ($p>0.05$). Compared with corresponding control group of miR-217 mimic NC group and si-CUL4B NC group, miR-217 mimic group and si-CUL4B group showed significantly decreased protein expressions of Wnt/β-catenin, Wnt1, Wnt3, Wnt3a and β-catenin (all $p<0.05$); while those expression levels were highly increased in miR-217 inhibitor group and oe-CUL4B group in relative to miR-217 inhibitor NC group and oe-CUL4B NC group (all $p<0.05$). In addition, compared with miR-217 mimic group, miR-217 mimic +oe-CUL4B group showed significantly increased protein expressions of Wnt/β-catenin, Wnt1, Wnt3, Wnt3a and β-catenin (all $p<0.05$). Corresponding results are shown in Figure 4. These results indicated that upregulated expression could inhibit the activation of Wnt/β-catenin signaling pathway via silencing the expression of CUL4B.

MiR-217 Targeted Downregulation of CUL4B Expression to Inhibit Cell Growth and IC50

The results (Figure 5) showed that compared with COC1 group, the cell proliferation activity of COC1/DDP group increased ($p<0.05$). Furthermore, compared with blank group, miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group, oe-CUL4B NC group and miR-217 mimic +oe-CUL4B group had no significant difference in the cell proliferation activity (all $p>0.05$); compared with miR-217 mimic NC group and si-CUL4B NC group, miR-217 mimic group and si-CUL4B group showed significantly decreased cell proliferation activity, respectively (all $p<0.05$); but the cell proliferation activity was evidently increased in miR-217 inhibitor group and oe-CUL4B group than that in miR-217 inhibitor NC group and oe-CUL4B NC group, respectively ($p<0.05$). Besides, compared with miR-217 mimic group, miR-217 mimic +oe-CUL4B group exhibited remarkably increased cell proliferation activity (all $p<0.05$).

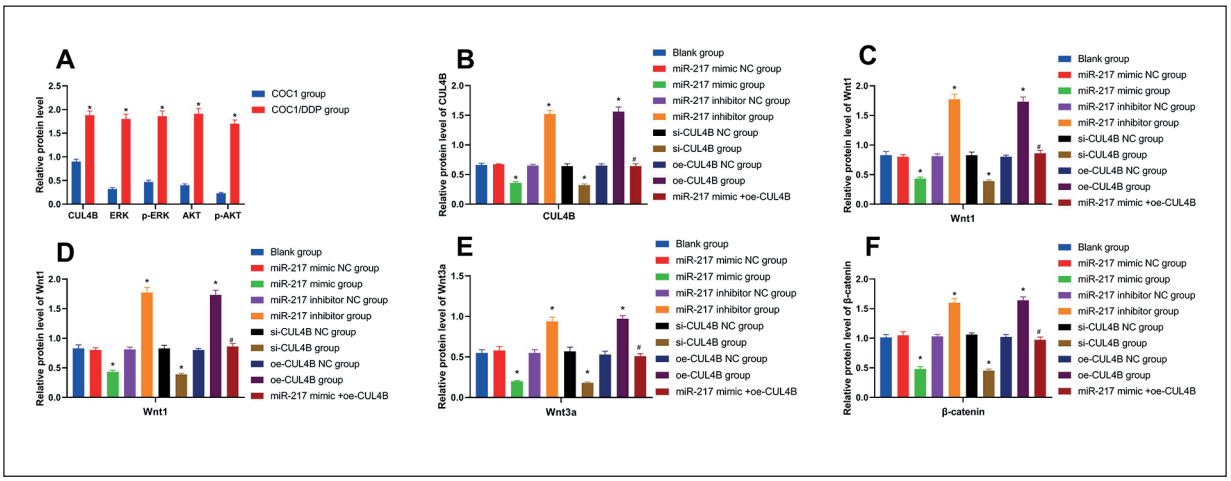

Figure 4. Protein expression level of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin in ovarian cancer cells after transfection in each group. **A**, The protein expressions of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; **B-F**, The protein expressions of CUL4B, Wnt1, Wnt3, Wnt3a and β-catenin in different transfection groups, *compared with blank group and corresponding NC groups, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$.

At the same time, compared with COC1 group, the IC50 value of cells was significantly upregulated in COC1/DDP group ($p<0.05$). No significant difference was found in IC50 value of miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group, oe-CUL4B NC group and miR-217 mimic +oe-CUL4B group when compared with that of the blank group (both $p>0.05$); meanwhile, compared with miR-217 mimic NC group and si-CUL4B NC group, there was significantly reduced IC50 value in miR-217 mimic group and si-CUL4B group, respectively (both $p<0.05$); but it was highly increased in miR-217 inhibitor group and oe-CUL4B group when compared with that in miR-217 inhibitor NC group and oe-CUL4B

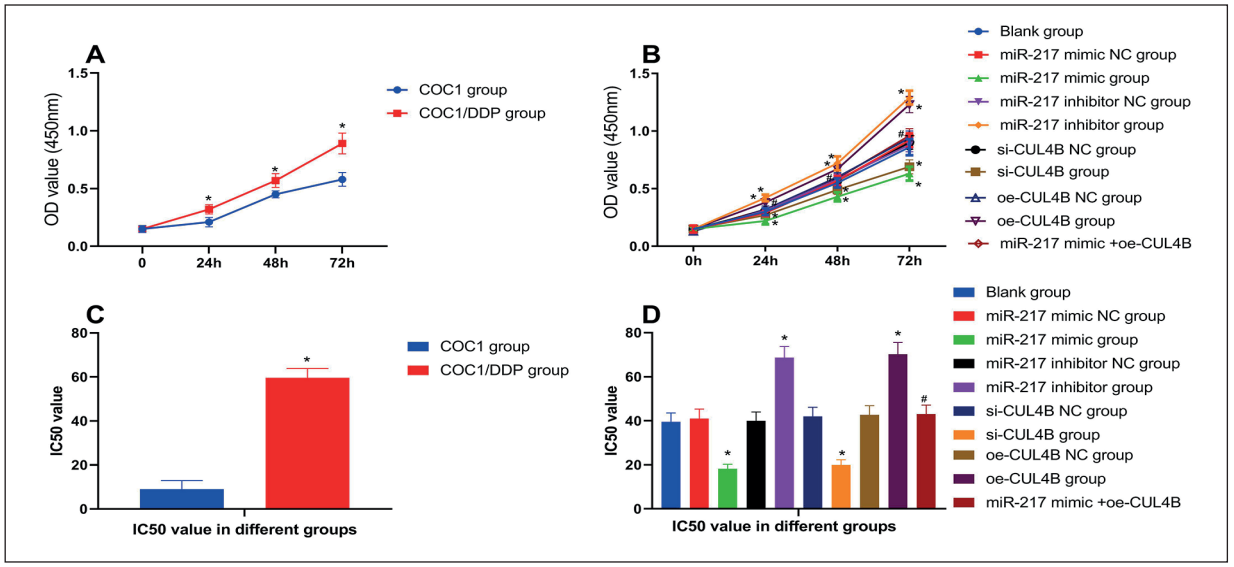

Figure 5. MTT assay results of the proliferation activity and IC50 value of cells in each group after transfection. **A**, The proliferation activity of cells in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; **B**, The proliferation activity of cells in different transfection groups, *compared with blank group and NC group, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$; **C**, The IC50 value of cells in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; **D**, The IC50 value of cells in different transfection groups, *compared with blank group and corresponding NC groups, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$.

NC group, respectively (p<0.05). In addition, there was an evident increase in IC50 value in miR-217 mimic +oe-CUL4B group than that in miR-217 mimic group (p<0.05).

MiR-217 Targeted Downregulation of CUL4B Expression to Inhibit Cell Invasion and Migration

As shown in Figures 6-7, the results of transwell showed that the invasion and migration ability of ovarian cancer cells were significantly enhanced in COC1/DDP group than that in COC1 group (p<0.05).

Furthermore, there was no significant difference in the invasion and migration ability of ovarian cancer cells among blank group, miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group, oe-CUL4B NC group and miR-217 mimic +oe-CUL4B group (all p> 0.05). Compared with miR-217 mimic NC group and si-CUL4B NC group, miR-217 mimic group and si-CUL4B group showed significantly suppressed cell invasion and migration, respectively (all p<0.05); while the ability of cell invasion and migration was promoted in miR-217 inhibitor group and oe-CUL4B group when compared with that in miR-217 inhibitor NC group and oe-CUL4B NC group, respectively (all p<0.05). While compared with miR-217 mimic group, miR-217 mimic +oe-CUL4B group revealed significantly enhanced cell invasion and migration abilities (all p<0.05). The results suggested that miR-217 could effectively inhibit the invasion and migration of ovarian cancer cells by upregulating the expression of CUL4B gene.

MiR-217 Targeted Downregulation of CUL4B Expression to Promote Cell Apoptosis

According to the results in Figure 8, the apoptosis of ovarian cancer cells was significantly decreased in COC1/DDP group than that in COC1 group (p<0.05). Furthermore, no significant difference was found in the cell apoptosis among blank group, miR-217 mimic NC group, miR-217 inhibitor NC group, si-CUL4B NC group, oe-CUL4B NC group and miR-217 mimic +oe-CUL4B group (all p> 0.05). Compared with miR-217 mimic NC group and si-CUL4B NC group, there was an increasing trend in the apoptosis rate of cell in miR-217 mimic group and si-CUL4B group, respectively (all p<0.05); while a decreased trend in the rate was observed in miR-217 inhibitor group and oe-CUL4B group than that in miR-217 inhibitor NC group and oe-CUL4B NC group, respectively (all p<0.05). Moreover, compared with miR-217 mimic group, there was an evident decrease in the apoptosis rate of miR-217 mimic +oe-CUL4B group (all p<0.05).

Discussion

To facilitate the development of molecular targeted therapy in human malignant tumors and to inhibit the progression and improve the prognosis of ovarian cancer patients, our study focused on the exploration of relevant molecules in the development of ovarian cancer. For the first time, our study identified that CUL4B was the direct target gene of miR-217, which provided reference

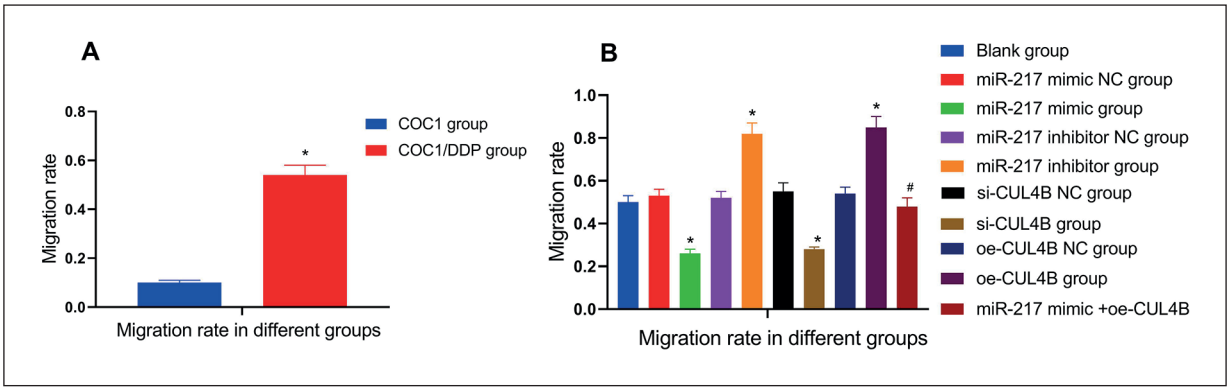

Figure 6. Cell scratch assay detection results of the cell migration in each group after transfection. **A**, Cell migration histogram in COC1/DDP group and COC1 group, *compared with COC1 group, p<0.05; **B**, Cell migration histogram in different transfection groups, *compared with blank group and corresponding NC groups, p<0.05; #compared with miR-217 mimic group, p<0.05.

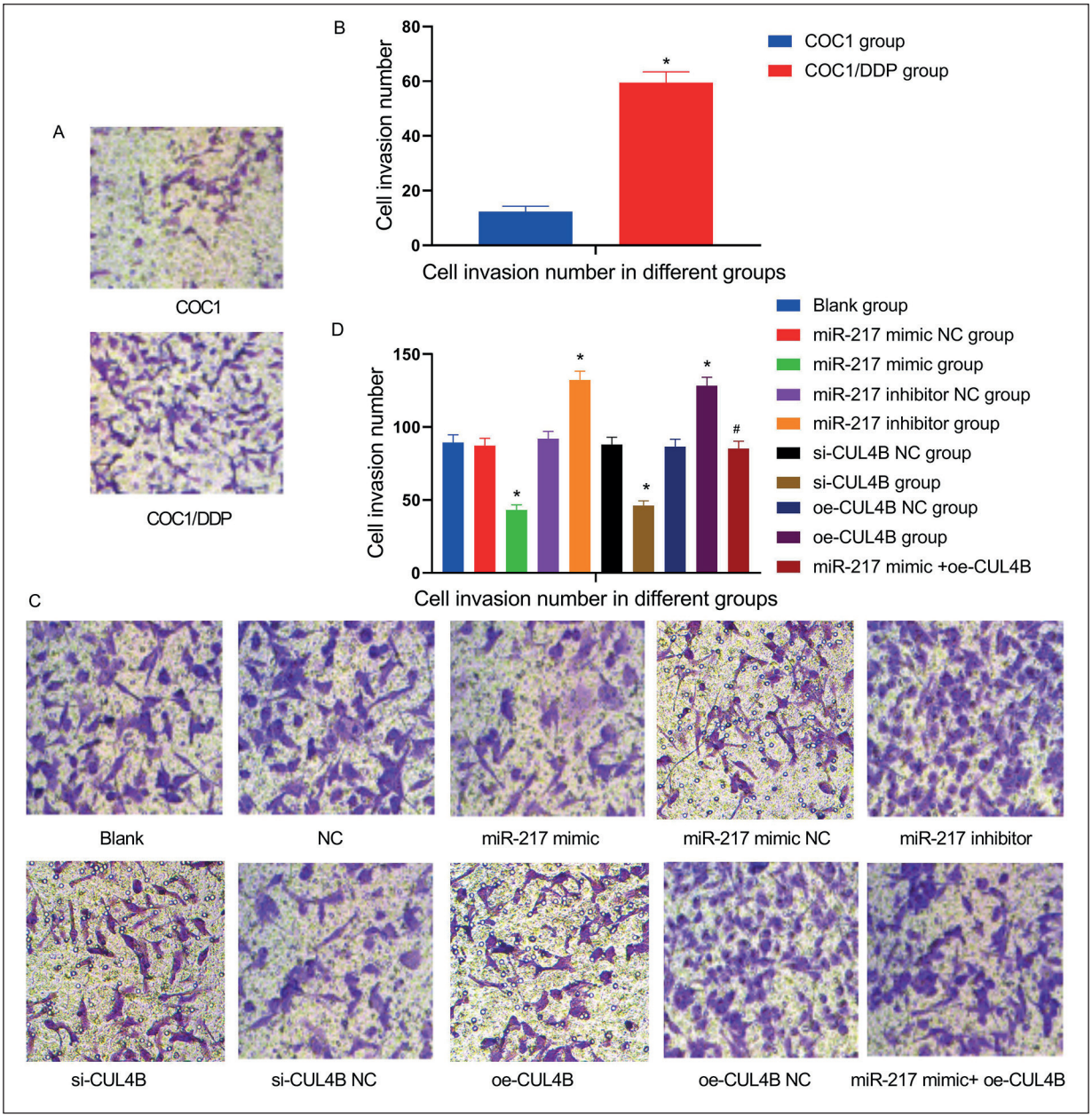

Figure 7. Transwell assay detection results of the cell invasion and migration in each group after transfection. **A**, Comparison of cell invasion between COC1/DDP group and COC1 group under light microscope (×200); **B**, Cell invasion histogram in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; Comparison of cell invasion in different transfection groups under light microscope (×200); **C**, Cell invasion histogram in different transfection groups, *compared with blank group and corresponding NC groups, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$.

for subsequent clarification of the mechanism of miR-217 regulating Wnt/β-catenin signaling pathway in ovarian cancer.

It has been documented concerning the separate function of miR-217 and CUL4B in the development of human malignancies. MiR-217 has been detected to be lowly expressed in various cancer such as colorectal cancer and hepatocellular cancer, but higher expressed in breast cancer, etc.[25-27]. It supports the abnormal expression of miR-217 in different types of tumors, and it deserves to be explored with respect to its expression in ovarian cancer. Meanwhile, CUL4B was also abnormally highly expressed in lung cancer, colorectal cancer, etc.[28-30]. Furthermore, it was reported that aberrant expression of miR-217

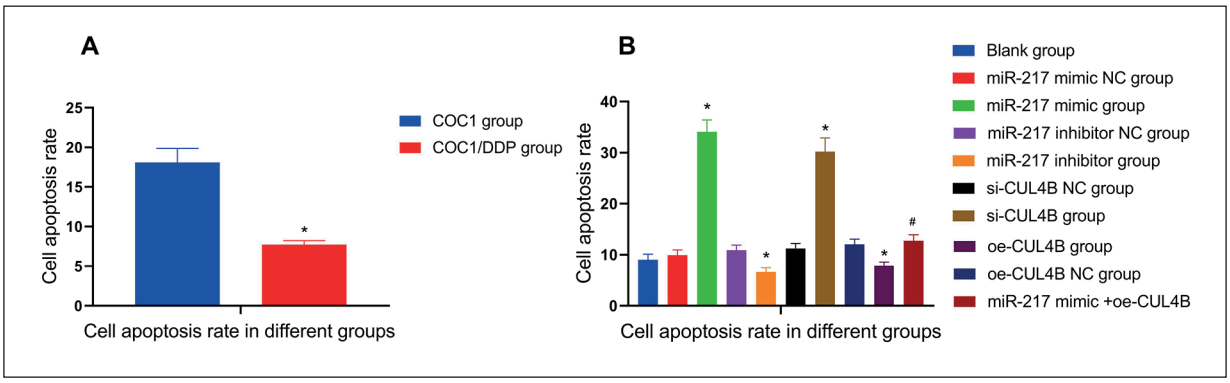

Figure 8. Flow cytometry detection results of the cell apoptosis in each group after transfection. **A**, Cell apoptosis histogram in COC1/DDP group and COC1 group, *compared with COC1 group, $p<0.05$; **B**, Cell apoptosis histogram in different transfection groups, *compared with blank group and corresponding NC groups, $p<0.05$; #compared with miR-217 mimic group, $p<0.05$.

can contribute to human cervical cancer growth, and it could regulate the expression of PTEN tumor suppressor to be involved in mediating cell proliferation and cervical cancer cell survival[31]. However, there are few studies related to the synergistic role of miR-217 and CUL4B in the progression of ovarian cancer, which was for the first time reported in the present study.

Significantly in our study, it was detected that the expressions of miR-217 and CUL4B in cisplatin-resistant ovarian epithelial cancer cell line COC1/DDP were significantly lower than that in cisplatin sensitive cell line COC1, suggesting that intervention in the expression of miR-217 and CUL4B may have a beneficial role in suppressing ovarian cancer. Accordingly, on the basis of different transfection protocols, our study found that the expression of miR-217 could be up-regulated while CUL4B was down-regulated after the overexpression of miR-217 but down-regulated after inhibited expression of miR-217. It indicated a successful transfection of miR-217, further identified the targeting relationship between miR-217 and CUL4B, and also highlighted the possible positive role of miR-217 overexpression in suppressing the expression of CUL4B. Meanwhile, there was no significant difference in the expression of miR-217 after silencing or overexpressing CUL4B, but with decreased or increased expression of CUL4B, respectively. It also proved that miR-217 targeted the expression of CUL4B and identified the smooth transfection of CUL4B. Besides, there was an increase in the expression of miR-217, but no significant change in CUL4B expression after combined transfection of miR-217 mimic and oe-CUL4B plasmids, which supplied a potential reverse demonstration concerning the relationship between miR-217 and CUL4B.

Furthermore, our study identified an increased trend in the protein expression of Wnt1, Wnt3, Wnt3a and β-catenin in cisplatin-resistant ovarian cancer cells than in cisplatin-sensitive cells, suggesting that the inhibited activation of Wnt/β-catenin signaling pathway may have a positive role in suppressing the development of ovarian cancer. Then, our experiment further verified an inhibited Wnt/β-catenin signaling pathway activation after overexpressing miR-217 and silenced expression of CUL4B gene, and a promoted activity of Wnt/β-catenin signaling pathway after downregulating or up-regulating corresponding expressions separately. Besides, the inhibited activation of Wnt/β-catenin signaling pathway could be reserved after combined treatment of miR-217 mimic and oe-CUL4B plasmids. The above results may provide potential evidence for the mechanism of miR-217 and CUL4B involved in the progression of ovarian cancer.

To check the rationality of the speculated mechanism of miR-217 and CUL4B in the development of ovarian cancer by regulating the activation of Wnt/β-catenin signaling pathway, our study explored the cisplatin resistance and biological behavior changes of ovarian cancer (e.g., proliferation, invasion, migration and apoptosis) in groups with different transfections. Consequently, it was found that in relative to COC1 group, the cisplatin resistance, proliferation, invasion and migration abilities of cells were enhanced while the apoptosis of cells was decreased in COC1/DDP group, which facilitated subsequent exploration of the speculated mechanism in improving the drug

resistance and biological behaviors of ovarian cancer cells. Specifically, similar to the aforementioned detected expression trends in each group, there were weakened cisplatin resistance, cell proliferation, invasion and migration abilities but improved cell apoptosis after overexpression miR-217 and silenced expression of CUL4B gene, but opposite trends after down-regulating or up-regulating corresponding expression separately. Meanwhile and remarkably, the beneficial improvement in cisplatin resistance, proliferation, invasion, migration and apoptosis was reversed after combined treatment of miR-217 mimic and overexpression-CUL4B plasmids when compared with that after overexpressing miR-217. It suggested the positive role of overexpressing miR-217 and silenced expression of CUL4B in ovarian cancer, and also proved the regulation of miR-217 on CUL4B since overexpression of CUL4B reserved the beneficial role of miR-217. Besides, according to the above investigation, we speculated that the positive roles of miR-217 and CUL4B may be related to the inhibited activation of Wnt/β-catenin signaling pathway.

With respect to the above, our study has the highlights of studying the mechanism of miR-217 in cisplatin resistance of ovarian cancer for the first time. Besides, it identified a potential regulatory axis of miR-217/CUL4B/Wnt/β-catenin signaling pathway in alleviating cisplatin resistance in ovarian cancer, and suppressing cell proliferation, migration and invasion, while promoting cell apoptosis, which may be related to the inhibited activation of Wnt/β-catenin signaling pathway. Accordingly, our research provides a new idea and direction for the study of cisplatin resistance and biological characteristics of ovarian cancer. However, it shall be noted that there are some limitations. There was no relevant experimental assay to explore the role of EMT in the development of ovarian cancer, and indeed EMT does plays an important role in the developmental process of this cancer. Besides, our study was a simple cell experiment with the selection of two human ovarian cancer cell lines COC1 (cisplatin sensitive) and COC1/DDP (cisplatin resistant). There was no design of an animal experiment to verify our findings in cell research. In future, our research group will continue to explore the mechanism of the occurrence, development, treatment and prognosis of ovarian cancer in a more comprehensive manner on the basis of an in-depth experimental design *in vivo* and *in vitro*.

Conclusions

To sum up, we report for the first time that CUL4B gene is the target gene of miR-217. MiR-217 can silence the expression of CUL4B, inhibit the activation of Wnt/β-catenin signaling pathway, enhance the cisplatin sensitivity and reverse drug resistance, inhibit cell invasion and migration, and promote cell apoptosis. For the first time, our study explores the role of miR-217-CUL4B-Wnt/β-catenin in the mechanism of cisplatin resistance, invasion and metastasis in ovarian cancer in accordance with reasonable biological prediction, comprehensive experimental design, which was achieved on the basis of human ovarian cancer cell lines COC1 (cisplatin sensitive) and COC1/DDP (cisplatin resistant) culture and treatment from the perspective of a reasonable hypothesis. It may provide e a theoretical basis for understanding the pathogenesis, diagnosis and prognosis of ovarian cancer, and provide additional therapeutic target for ovarian cancer. Further animal experiments and clinical trials are deserved to be performed to promote an in-depth exploration of the mechanism axis of miR-217-CUL4B-Wnt/β-catenin in ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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