miR-200b and miR-200c co-contribute to the cisplatin sensitivity of ovarian cancer cells by targeting DNA methyltransferases

JUE LIU¹*, XIAOBO ZHANG²*, YULIANG HUANG¹, QUNFENG ZHANG¹, JIANBIN ZHOU¹, XIAODI ZHANG¹ and XIAOXU WANG³

¹Department of Obstetrics and Gynecology, The Second Affiliated Hospital, University of South China, Hengyang, Hunan 421001; ²Department of Geriatric Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008; ³Department of Joint Surgery, The Second Affiliated Hospital, University of South China, Hengyang, Hunan 421001, P.R. China

Received December 13, 2016; Accepted August 16, 2018

DOI: 10.3892/ol.2018.9745

Abstract. Cisplatin is a first-line chemotherapy drug that is commonly used in the treatment of epithelial ovarian cancer (EOC). However, insensitivity to cisplatin markedly influences the outcomes of chemotherapy. MicroRNAs (miRNAs/miRs) have been demonstrated to modulate drug resistance in a number of types of cancer. The aim of the present study was to investigate the key miRNAs involved in modulating drug resistance in ovarian cancer cells. miR-200b and miR-200c were identified to be frequently deregulated in ovarian cancer. Upregulation of miR-200b and miR-200c promoted EOC cell death in the presence of cisplatin. Upregulation of miR-125b-5p significantly decreased tumor growth in combination with cisplatin in a mouse model. Significantly, miR-200b and miR-200c reversed cisplatin resistance by targeting DNA methyltransferases (DNMTs) (directly targeting DNMT3A/DNMT3B and indirectly targeting DNMT1 via specificity protein 1). These results indicate that miR-200b- and miR-200c-mediated regulation of DNMTs serves a crucial function in the cellular response to cisplatin. miR-200b- and miR-200c-mediated downregulation of DNMTs may improve chemotherapeutic efficacy by increasing the sensitivity of cancer cells and thus may have an impact on ovarian cancer therapy.

Introduction

With an estimated 21,290 novel cases of epithelial ovarian cancer (EOC) and 14,180 cases of associated mortality in 2015, EOC is the fifth leading cause of cancer-associated mortality in women in the USA (1). Owing to a lack of effective biomarkers and disease-specific symptoms, particularly for early-stage EOC, a marked proportion of patients are not diagnosed until an advanced stage. Cytoreductive surgery with cisplatin-based chemotherapy is the preferred treatment. However, resistance to chemotherapy leads to a dismal prognosis (2,3). Therefore, an extensive understanding of the molecular mechanisms in EOC is crucial.

Previous studies have emphasized that epigenetic modifications, particularly DNA hypermethylation, may be among the molecular mechanisms underlying acquired resistance to cisplatin (4,5). Multiple DNA methylation changes in the cancer methylome are associated with the acquisition of drug resistance (5-7). A significant upregulation of DNA methyltransferases (DNMTs) has been observed in cisplatin-resistant ovarian cancer (8). Three DNMTs have been identified in humans: DNMT1, DNMT3A and DNMT3B. DNMT1 is the most abundant DNMT in mammalian cells, and is the key enzyme for the maintenance of hemimethylated DNA during DNA replication and the development and differentiation of somatic cells (9); it serves an important function in the silencing of several tumor suppressor genes and accumulates in the promoter regions of these genes (10-12). Decitabine is one of the most widely used DNMT inhibitors in research and in cancer therapy. Although it can have a major impact in combination with other chemotherapeutic drugs, its narrow therapeutic window and effective dosage limit its clinical use (13).

MicroRNAs (miRNAs/miRs) are a class of short non-coding RNAs, between 19 and 25 nucleotides in length, that regulate gene expression by targeting miRNAs and that have functions in multiple physiological and pathological functions (14). It has been identified that ~30% of genes are regulated by miRNAs (15), and >60% of protein-coding genes are computationally predicted as being miRNA targets (16). miRNAs may be controlled or may be used to control target genes in aberrant DNA hypermethylation.

One particular miRNA family, the miRNA-200 family, regulates DNA methylation in a number of types of cancer (12,17). Ectopic overexpression of the two miRNAs increased the sensitivity of the resistant ovarian cancer cells...
to cisplatin by promoting apoptosis by directly suppressing DNMT3A and DNMT3B, and also indirectly decreasing the expression of DNMT1 via the downregulation of specificity protein (Sp)1, a transactivating factor of the DNMT1 gene (12,18). This provides attractive novel avenues for the development of therapeutic approaches based on the molecules involved in DNA methylation.

Materials and methods

Ethical approval. The present study was approved by the Institutional Review Board of The Second Affiliated Hospital of South China University (Hengyang, China). All tissues were obtained following written informed consent from the patients.

Patient samples and cell lines. Frozen human primary ovarian tumor and corresponding adjacent non-cancerous tissues used in the present study were obtained from patients diagnosed between October 2007 to September 2014 who underwent radical resection at The Second Affiliated Hospital, University of South China. The average age was 55±6.5 years. Patients who received some form of chemotherapy or radiotherapy prior to surgery were excluded from the study. The human ovarian cancer cell lines and human immortalized ovarian surface epithelial (HIOSE-80 and MCC-3) cell lines used were described previously (19). The human ovarian cancer cell lines SKOV3, A2780CP and A2780, and human ovarian surface epithelial cell lines were obtain from the American Type Culture Collection (Manassas, VA, USA). OV119 cells were purchased from the Beijing Institute for Cancer Research (Beijing, China). HIOSE-80 cells were cultured with 199/MDCB 105 (1:1) medium (Sigma; Merck KGaA, Darmstadt, Germany) supplemented with 5% fetal bovine serum (FBS). All other cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS and 1% penicillin/streptomycin.

Transfections and luciferase assay. Cells were seeded in 6-well plates at 1×10⁵ cells/well followed by culture for 24 h and transfection with 20 nmol/1 miR-200b mimic, 5'-CAUCUUACUUGGCAGCAUUGGA-3', miR-200c mimic, 5'-CGUCUUACCAGCAGUGUUGG-3' or negative control mimics (NC) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The NC consisted of synthetic double-stranded oligonucleotides that do not target any mRNA. The effect of the mimics was determined in triplicate for 24 h post-transfection.

MTT assay. Non-transfected or transfected cells were re-seeded in 96-well plates; 24 h later, freshly prepared cisplatin (Sigma; Merck KGaA) at 20 µM cisplatin treatment was added, and the cells were cultured for an additional 48 h. Cell viability was determined using an MTT assay (Thermo Fisher Scientific, Inc.). The resulting absorbance of each well was determined at 492 nm on a spectrophotometer. At least three independent experiments were performed in quadruplicate.

In situ hybridization (ISH) and immunohistochemistry (IHC) assays. ISH procedures were carried out as described previously (20). miR-200b and miR-200c miRCURY locked nucleic acid custom detection probes miR-200b mimic, 5'-CAUCUUACUUGGCAGCAUUGGA-3', miR-200c mimic, 5'-CGUCUUACCAGCAGUGUUGG-3' (Qiagen, Inc., Valencia, CA, USA) were used for ISH. Hybridization, washing and scanning were performed according to the manufacturer's protocol. Paraffin-embedded blocks of tumors were sectioned into 5-µm slices, and the IHC protocol was performed as described previously (21). Staining intensity was scored as follows: >0 and ≤1, no staining; >1 and ≤2, weak staining; >2 and ≤3, medium staining; and >3 and ≤4, strong staining. The proportion of positive cells was divided into four groups: 0-25, 26-50, 51-75 and 76-100%. The final score was determined by multiplying the intensity score and the quantity score; the maximum was 4, and the minimum was 0. All specimens were evaluated by at least two blinded pathologists. Expression scores ≥2 were classified as high expression, and scores <2 were classified as low expression.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT of specific miRNAs (from 10 ng of total RNA) was performed using the real-time loop primers for each type of miRNAs and the TaqMan miRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. cDNA obtained from this step was used for quantitative TaqMan PCR using the real-time primers provided. Reverse-transcribed cDNA was synthesized with random primers or miRNA-specific stem-loop primers. LightCycler Fast Start DNA Master SYBR Green Mix (Roche Diagnostics GmbH, Mannheim, Germany) was added to each PCR reaction along with cDNA and 1 pmol primer in a total volume of 10 µl. The primer sequence for miR-200b was as follows: Forward, 5'-CACACTGAAATCCGTTCAGCTTC-3' and reverse, 5'-CTAACT. The primer sequence for miR-200b mimics was sense, 5'-UUCUCGGACGUGUCACGUTT-3' and anti-sense, 5'-ACGGUGCACGUUGAGAATT-3'. The PCR thermocycling conditions were as follows: One cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 12 sec and 62˚C for 35 sec, 94°C for 5 min, 50°C for 10 min and finally 1 cycle at 62-95°C for 15 sec. The relative expression level of each RNA was quantified using the 2−ΔΔCq method (22). CR was performed in triplicate using a standard SYBR Green PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Western blot analysis. The cells were lysed in radioimmunoprecipitation assay buffer in the presence of proteinase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) on ice. The lysates were centrifuged at 12,000 x g, for 10 min at 4°C, and SDS gel loading buffer was added. A total of 20 µg of protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 10% skimmed milk powder with PBS followed by incubation for 1 h at room temperature with the following primary antibodies (1:500): Anti-DNMT1 (cat. no. sc-10222; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-DNMT3A (cat. no. sc-20703; Santa Cruz Biotechnology, Inc.), anti-DNMT3B (cat. no. sc-10236; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (cat...
Following washing 3 times with 0.1% PBS, the membranes were incubated with rabbit anti-mouse HRP (cat. no., BA1058) and goat anti-rabbit HRP (cat. no., BA1058) secondary antibodies purchased from Wuhan Boster Biological Technology, Ltd., (Wuhan, China) at a dilution of 1:5,000 for 1 h at room temperature. Protein band were visualized using enhanced chemiluminescence western blot detection reagents (New England and Biolabs, Inc., Ipswich, MA, USA), according to the manufacturer's protocols. The protein levels were normalized to the levels of GAPDH and quantifed by a Bio Image Intelligent Quantifier 1-D 2.2.1 (Nikon Corporation, Tokyo, Japan), according to the manufacturer's protocols.

**Flow cytometry-based apoptosis.** Cells were cultured in cisplatin-containing medium and incubated for 48 h at room temperature. Following incubation, the cells were harvested and stained with annexin V-fluorescein isothiocyanate and propidium iodide. The mixture was incubated at room temperature in the dark for 15 min and analyzed by fluorescence-activated cell sorting (FACS), using BD FACSCanto I. Flowjo 7.6 software (BD Biosciences, Franklin Lakes, NJ, USA).

**Nude mouse model.** A total of ~10^7 cells were injected intraperitoneally into nude mice. Cells were transected with 20 nmol/l miR-200b mimic, miR-200c mimic or NC using Lipofectamine® 2000 respectively 48 h before being injected into mice. After 1 week, cisplatin therapy was initiated at a dose of 5 mg/kg twice weekly. Tumor size was calculated every 4 days according to the following formula: Tumor size=(π/6) x larger diameter x (smaller diameter)^2. After 4 consecutive weeks of therapy, the mice were sacrificed, and the wet weights of the tumors were determined.

**Luciferase reporter assay.** Cells were co-transfected with 500 ng PGL3-DNMT1/DNMT3A/DNMT3B-WT or PGL3-DNMT1/DNMT3A/DNMT3B-Mut constructs (both from GeneCopoeia, Inc., Rockville, MD, USA) with miR-200b and miR-200c mimic or NC. Each sample was co-transfected with pRL-TK plasmid (GeneCopoeia, Inc.) to determine the transfection efficiency. Luciferase activity was examined 48 h after transfection using a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

**Statistical analysis.** All statistical analyses were performed using SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences between samples were analyzed using two-tailed Student's t-test, and the comparisons of multiple groups were performed by one-way analysis of variance and Bonferroni's post hoc test. Spearman's correlation analysis was used to evaluate the association between miR-200b/c and DNMT1/3A/3B. P<0.05 was considered to indicate a statistically significant difference.
miR-200b and miR-200c are downregulated in cisplatin-resistant ovarian cancer. The expression levels of miR-200b and miR-200c were determined in 93 ovarian tumors and 32 normal ovarian tissues using ISH, and it was identified that miR-200b and miR-200c were downregulated in ovarian tumors compared with normal tissues. Among the 93 patients with primary ovarian tumors, 35 had recurrent (chemoresistant) ovarian cancer. ISH analysis revealed that the levels of miR-200b and miR-200c were low or undetectable in these recurrent ovarian cancer tissues (Table I). These results indicated that miR-200b and miR-200c were significantly downregulated in chemoresistant ovarian tumors compared with normal tissues, and implied that miR-200b and miR-200c may be involved in cisplatin resistance in patients with ovarian cancer.

To further verify the biological function of miR-200b and miR-200c in human ovarian cancer, their expression was confirmed in ovarian cancer cell lines using RT-qPCR analysis. The expression of miR-200b and miR-200c was downregulated in cancer cell lines compared with immortalized human surface epithelial cell lines HIOSE-80 and MCC-3, and A2780CP cells had the lowest expression compared with the other ovarian cancer cell lines investigated (Fig. 1). These results suggested that the expression of miR-200b and miR-200c is significantly altered in EOC, and that the low expression is associated with poor prognosis of patients with EOC.

Overexpression of miR-200b and miR-200c increases the cisplatin sensitivity of ovarian cancer cells. It has been identified that A2780CP cells are markedly resistant to cisplatin treatment, therefore it was investigated whether miR-200b and miR-200c served any function in the sensitivity of cisplatin treatment in EOC. miR-200b mimic, miR-200c mimic and NC were transfected into the ovarian cancer cell line A2780CP. Total RNA was extracted, and the transfection efficiency was evaluated at 48 h after transfection (Fig. 2A). No significant alteration in cell proliferation among the control, miR-200b and miR-200c groups was identified. This was consistent with the results of a previous study in hepatocellular carcinoma (23). The transfected cells were then exposed to various concentrations of cisplatin for 48 h and viability was determined using an MTT assay. It was identified that the half-maximal inhibitory concentration (IC$_{50}$) of cisplatin was lower in cells that had been transfected with miR-200b and miR-200c compared with the cells that had been transfected with the NC (Fig. 2B). These results indicated that miR-200b and miR-200c are involved in cisplatin sensitivity in EOC cells, and the overexpression of miR-200b and miR-200c markedly reversed the cisplatin sensitivity of A2780CP cells. To test whether the effect of miR-200b and miR-200c on cisplatin sensitivity was associated with the duration of treatment, a cell survival assay was performed at 24, 48 and 72 h after transfection. The results indicated a stable effect of the two miRNAs following transfection (Fig. 2C). Cell death was determined using annexin V staining and FACS analysis.

Overexpression of miR-200b and miR-200c did not affect cell death, and the overexpression of the two miRNAs in these cells led to marked cell death when the cells were treated with cisplatin (Fig. 2D). These results indicated that miR-200b and miR-200c are involved in cisplatin sensitivity in EOC cells.
Overexpression of miR-200b and miR-200c reverses cisplatin resistance in vivo. As the overexpression of miR-200b and miR-200c significantly decreased the IC_{50} of cisplatin, the therapeutic potential of miR-200b and miR-200c was investigated in vivo. Nude mice were subcutaneously injected with A2780CP cells transfected with miR-200b, miR-200c mimic or NC. Tumor volumes were determined. Transduction of miR-200b and miR-200c did not affect tumor growth in vivo. Cisplatin therapy was administered at a dose of 5 mg/kg twice weekly. After 4 consecutive weeks of treatment, the mice were sacrificed. The tumors were excised, and the wet weights of the tumors were determined. Representative images of xenografts are shown. (A) Growth of subcutaneous tumors following injection of NC- and miR-200b/miR-200c mimic-transfected A2780CP cells followed by treatment with cisplatin or DMSO for 4 weeks. Tumor size was assessed every 4 days. (B and C) After 4 consecutive weeks of therapy, the mice were sacrificed, and the wet weights of the tumors were determined. Representative images of xenografts are shown. (B) Summary of tumor weights in nude mice. Results are presented as the mean ± standard error of three independent experiments. miR, microRNA; NC, negative control mimics; DMSO, dimethylsulfoxide; cis, cisplatin.

Overexpression of miR-200b and miR-200c directly target DNMT3A and DNMT3B, and indirectly target DNMT1 in ovarian cancer cells. Bioinformatics analyses predicted that DNMT3A and DNMT3B are the potential targets of miR-200b and miR-200c, and it has been demonstrated that DNMT3A and DNMT3B are true targets of in human gastric cancer (24). A dual-luciferase reporter assay was performed in A2780CP cells. Co-transfection of miR-200b or miR-200c and the reporter plasmids revealed that the introduction of miR-200b/miR-200c significantly suppressed the luciferase activity of the vectors containing the 3'-untranslated region (UTR) of DNMT3A and DNMT3B, but not of those containing mutations in the mRNA-binding site of DNMT3A/DNMTB. It was identified that miR-200b and miR-200c directly targeted the 3'-UTR of DNMT3A and DNMT3B, respectively (Fig. 4A). Previous studies have revealed that Sp1 positively regulates DNMT1 by increasing the activity of the DNMT1 promoter (24). In the present study, it was confirmed whether miR-200b and miR-200c downregulated DNMT1 indirectly via Sp1 in ovarian cancer cells. To this end, the 3'-UTR of Sp1 was cloned into a luciferase reporter vector, and the results revealed that miR-200b and miR-200c bound directly to Sp1 and markedly decreased luciferase activity (Fig. 4A). Therefore, the transfection of miR-200b mimic and miR-200c mimic into A2780CP cells resulted in a marked decrease in DNMT1, DNMT3A and DNMT3B protein levels compared with the transfection of NC (Fig. 4B).

To investigate whether miR-200b and miR-200c negatively regulate their target genes in clinical samples, endogenous DNMT1, DNMT3A and DNMT3B expression was determined in human ovarian cancer tissues. Immunohistochemical staining of DNMT1, DNMT3A and DNMT3B revealed an increased expression of these DNMTs in EOC compared with the neighboring normal tissues (Fig. 4C). An inverse correlation between miR-200b/miR-200c and DNMT1/DNMT3A/DNMT3B was identified in randomly selected human ovarian cancer sections (Fig. 4D). These results further supported the regulation of DNMT1, DNMT3A and DNMT3B by miR-200b and miR-200c, and indicated the significance of miR-200b and miR-200c as biomarkers in the progression of EOC.

Discussion

Cisplatin is one of the most widely used first-line chemotherapy drugs for treating advanced-stage malignancies, such as testicular, cervical and non-small cell lung cancer. It has been called a 'platinum chemotherapeutic agent' (25). Numerous studies with cisplatin-based combination therapies have been performed over the last 30 years. Despite a marked initial response, the efficacy of cisplatin is significantly hindered by the development of resistance during treatment. Therefore, patients with advanced carcinoma are not eligible for standard treatment with cisplatin-based chemotherapy (26). Furthermore, combination therapies of cisplatin with other drugs or mechanisms of resistance have been considered to overcome drug resistance and to decrease toxicity.

Although multiple mechanisms that mediate intrinsic or acquired resistance to cisplatin have been recognized, alterations in the DNA repair capacity of damaged cells are now being recognized as being important in regulating the resistance to cisplatin (27-29). Consequently, alterations in DNA repair pathways have been implicated in cisplatin resistance. Strategies
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Figure 4. miR-200b and miR-200c target DNMT1 and DNMT3A/3B in human ovarian cancer. (A) miR-200b and miR-200c inhibit the reporter activity of wt, but not mut DNMT1/3A/3B-3'-UTR-untranslated region. An empty luciferase reporter construct was used as the negative control. *P<0.05 vs. NC. (B) A2780CP cells was transfected with NC, miR-200b and miR-200c mimics for 24 h. Expression of DNMT1, DNMT3A and DNMT3B in the cells was determined by western blotting (normalized to GAPDH). (C) Ovarian cancer specimens were analyzed by ISH and immunohistochemical staining, and the representative miR-200b, miR-200c, DNMT1, DNMT3A, and DNMT3B expression are presented. (D) Analysis of immunohistochemical data by linear regressions and inverse correlations of miR-200b/c with DNMT1, DNMT3A, and DNMT3B in human ovarian cancer. miR, microRNA; DNMT, DNA methyltransferase; wt, wild-type; mut, mutated; NC, negative control mimics.

For overcoming cisplatin resistance are urgently required in cancer therapy. Substantial changes in DNA methylation have previously been reported to occur during the acquisition of cisplatin resistance. DNA methylation is an epigenetic modification that is mediated by DNMTs. DNMT1 is the most abundant DNMT in mammalian cells and the key enzyme for the maintenance of hemimethylated DNA during DNA replication and tumorigenesis. DNMTs have also been identified to be overexpressed in a number of malignancies (30-32). In previous studies, DNMT1 and DNMT3A/DNMT3B were identified to be upregulated in a cisplatin-resistant ovarian cancer cell line, suggesting a common regulatory pathway for the expression of DNMT genes (25,26). Several human miRNAs, including those of the miR-29 family, miR-148 and miR-143, have been identified to be frequently downregulated in human cancers, and to lead to the increased expression of DNMT1 and DNMT3A or DNMT3B because they directly target the 3'-UTR of DNMTs (33). miR-200a, miR-200b, miR-200c, miR-411 and miR-429 belong to a cluster of miRNAs that are markedly associated with epithelial-mesenchymal transition (EMT), where miR-200b and miR-200c are identified as critical regulators of tumor invasion, metastasis
and chemosensitivity (34). miR-200b and miR-200c are known to inhibit the translation of EMT activators, particularly zinc finger E-box-binding homeobox factors, and EMT activators, thereby inducing mesenchymal-epithelial transition (MET) (35-38). Thus, miR-200 family members, in particular miR-200b and miR-200c, control crucial cellular processes, such as motility and stemness, and their own regulators also serve an important function in these processes. The results of the present study suggested that the upregulation of miR-200b and miR-200c may lead to the repression of DNMT1 and DNMT3A/DNMT3B and in turn contribute to the sensitivity of EOC cells to cisplatin.

In the present study, the function of miR-200b and miR-200c in cisplatin resistance was characterized, and it was identified that miR-200b and miR-200c increased cisplatin sensitivity principally through the direct downregulation of DNMT3A/DNMT3B and the indirect downregulation of DNMT1 by targeting Sp1. Sp1 and Sp3 have been reported to increase the activity of the DNMT1 promoter by physically binding to it in mouse NIH3T3 cells (39). In addition, previous studies have identified that wild-type p53 was able to negatively regulate the DNMT1 gene by binding to the Sp1 protein at their binding sites on the DNMT1 promoter; furthermore, wild-type p53 was identified to modulate Sp1 to act as a co-repressor with histone deacetylase (HDAC)1, HDAC6 and retinol-binding protein 2 lysine demethylase to suppress the expression of the DNMT1 gene when the level of Sp1 protein was low (12). On the other hand, p53 has been confirmed to be a transcriptional activator of the gene encoding miR-200c, and its clinical relevance, as validated in human breast cancer, revealed an association of mutant p53 expression with decreased levels of miR-200c (40). Results from a previous study (40) also support the hypothesis that alterations in p53 may influence the sensitivity to cisplatin-based chemotherapy because p53 interacts with cisplatin-damaged DNA molecules.

In conclusion, the results of the present study suggested a possible mechanism by which miR-200b and miR-200c enhance cisplatin sensitivity by promoting apoptosis, and suggested their potential use as therapeutic targets for overcoming cisplatin resistance in ovarian cancer.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed in the present study are included in this article.

Authors' contributions

JL and XXW conceived the study and wrote the manuscript. JL, XBJ, YLH, QFZ, JBZ and XDZ performed experiments, collected data, and analyzed data. XXW, YLH, QFZ and JBZ reviewed, and revised manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of The Second Affiliated Hospital of South China University (Hengyang, China). All tissues were obtained following written informed consent from the patients.

Patient consent for publication

Written informed consent was obtained by the patients and/or guardians.

Competing interests

The authors declare that they have no competing interests.

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