ARID1A gene silencing reduces the sensitivity of ovarian clear cell carcinoma to cisplatin

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Abstract. In ovarian clear cell carcinoma (OCCC), the mutation rate of the AT-rich interaction domain 1A (ARID1A) gene is 46-57%. However, the effects of ARID1A gene silencing by small interfering RNA (siRNA) on the sensitivity of OCCC to cisplatin have not been investigated. Thus, this study aimed to elucidate the association between ARID1A gene silencing and drug resistance in OCCC. Three pairs of ARID1A gene siRNA fragments (siRNA-1, siRNA-2 and siRNA-3) were designed and transiently transfected into ES2 OCCC cells using RNAi Max reagent. Western blotting results demonstrated that the transfection reduced ARID1A protein expression levels, with the siRNA-3 group having the lowest levels. The IC_{50} value, determined using a Cell Counting kit-8 assay, was significantly increased by siRNA-3 transfection compared with that in blank control and negative control groups. The cell survival rate following treatment with 50 μM cisplatin for 48 h was significantly increased in the siRNA-3 group compared with the blank control and negative control groups. Flow cytometric analysis revealed that the apoptosis rate for cisplatin-treated cells was significantly lower in cells with siRNA-3 transfection than in those without, and the apoptosis rate in siRNA-3-transfected cells was lower than that in the negative control group. Western blot analysis showed that the expression level of AKT in cisplatin-treated cells was significantly decreased compared with that in the negative control group, and the AKT expression level in cisplatin-treated cells was significantly higher with siRNA-3 transfection than without. Therefore, the results demonstrated that ARID1A siRNA efficiently decreased ARID1A expression, which reduced cisplatin chemosensitivity and cell apoptosis in ES2 OCCC cells via the regulation of AKT expression.

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Introduction

Ovarian clear cell carcinoma (OCCC) is a rare type of epithelial ovarian cancer with high degree of malignancy, and its incidence is 5-11% of ovarian epithelial tumors. OCCC is reported to occur at an earlier age than serous ovarian cancer, with a median age at diagnosis of 55 years compared with 64 years (1). Although low-stage OCCC has a relatively good prognosis, advanced-stage OCCC has a significantly lower overall survival rate (2,3). The treatment of OCCC mainly comprises surgery combined with radiotherapy and chemotherapy in a comprehensive treatment program. However, the resistance to platinum-based traditional chemotherapy is very common in the clinic, so the prognosis is poor (4-7).

Chromatin remodeling, including the synthesis, transcription and repair of DNA, is important in cell nuclear activities. Genetic mutation of the chromatin remodeling complex has been identified as a mechanism of tumor occurrence and development (8). The AT-rich interaction domain 1A (ARID1A) as a non-catalytic subunit of the chromatin remodeling complex, has the ability to combine with DNA or protein. Genetic mutations of ARID1A in various tumors are considered to be associated with the biological behavior, treatment and prognosis of the tumor (9,10). In OCCC, the mutation rate of the ARID1A gene has been found to be 46-57% (11). However, whether such a high mutation rate is associated with the resistance of OCCC to chemotherapy remains unclear and requires investigation. Therefore, the aim of the present study was to evaluate the sensitivity of the OCCC cell line ES2 to cisplatin following silencing of the ARID1A gene and to investigate the possible mechanism.

Materials and methods

Reagents and antibodies. Lipofectamine RNAi Max reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-ARID1A (ab50878) and anti-β-actin (ab134032) mouse monoclonal antibodies, and anti-AKT (ab179463) rabbit monoclonal antibodies were purchased from Abcam (Cambridge, UK).

Design and synthesis of small interfering RNA (siRNA) sequences. The three pairs of ARID1A gene siRNA interference fragments (siRNA-1, siRNA-2 and siRNA-3), and one

pair of sequences unrelated to ARID1A (negative control, NC) were designed and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequences used were as follows: siRNA-1 forward, 5'-GCCCUGAACAAUAACCUCATT-3' and reverse, 5'-UGAGGUUAUUGUUCAGGGCTT-3'; siRNA-2 forward, 5'-CCAGUCCAAUGGAUCAGAUTT-3' and reverse, 5'-AUCUGAUCCAUUGGACUGGTT-3'; siRNA-3 forward, 5'-CAGCUUGCCUGAUCUAUCUTT-3' and reverse, 5'-AGAUAGAUCAGGCAAGCUGTT-3'; NC forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'.

Cell culture and transfection. A ES2 cell line was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China) and maintained in McCoy's 5A culture medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany). Cultured cells were incubated in a humidity chamber (Thermo Fisher Scientific, Inc.) containing 5% CO_2 at 37˚C. For transfection, Lipofectamine RNAi Max was mixed with the aforementioned siRNA, according to the manufacturer's instructions. The solutions were subsequently combined with ES2 cells in 6-well culture plates at a density of $3.0x10^5$ cells/well. In the blank control group, cells were treated with the transfection reagent only (Lipofectamine RNAi Max); however, in the normal control group, the cells did not undergo any treatment.

Western blot analysis. Cells were washed twice with PBS and lysed in a buffer containing 50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 10% glycerol and 0.5% NP-40 and protease inhibitor cocktail (Sigma-Aldrich; Merck Millipore). Then, cells were centrifuged for 15 min at 4˚C at 14,000 x *g*. Protein concentration was determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (50 μg) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked using 5% skimmed milk powder at room temperature for 1 h. Then, the membranes were incubated with anti-ARID1A (1:500), anti-AKT (1:1,000) and anti-β-actin (1:5,000) primary antibodies at 4˚C overnight, followed by incubation with a horseradish peroxide-conjugated mouse IgG secondary antibody (ab190475; 1:10,000; Abcam) or rabbit IgG secondary antibody (ab190495; 1:10,000; Abcam) at room temperature for 2 h. Proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare Life Sciences, Piscataway, NJ, USA), and the protein band intensity was quantified via densitometric analysis using Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cisplatin treatment and cell viability assay. Cell inhibition rate and cell viability (IC_{50}) values for cisplatin (Sigma-Aldrich; Merck Millipore) were determined using the Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology). Cells at a density of $3.0x10^3$ cells/well were plated in 100 μl medium/well in 96-well culture plates. Following overnight incubation at 37˚C and 5% CO_2, cells were transfected with siRNA in the presence of Lipofectamine RNAi Max. After 24 h of incubation, cells were exposed to cisplatin at a range of concentrations (20, 40, 80, 120 and 160 μM). Each concentration of drug was added to duplicate wells. Following 48 h of incubation, 10 μl/well CCK-8 reagent was added directly to the medium and the plates were incubated for 1 h. The absorbance values were read on a SpectraMax M3 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and converted to percentage cell viability in corresponding matched CCK-8-treated cells, which were designated as 100% viable. IC_{50} values (concentration of drug that results in a reduction of the absorbance signal by 50% compared with the CCK-8-treated control) were obtained from nonlinear regression analysis of concentration-effect curves using SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). The IC_{50} value in the negative control group was ~52 μM. Then, 50 μM cisplatin was added to the plates after transfection, the cells were incubated for 48 h and the cell survival rate of each group was calculated.

Flow cytometry assay. ES2 cells ($3x10^5$/well) were plated in 2 ml medium/well in 6-well culture plates. Following overnight incubation at 37˚C and 5% CO_2, cells were transfected with NC or siRNA-3 siRNA in the presence of Lipofectamine RNAi Max. Following incubation for 24 h, 50 μM cisplatin was added to the plates and the cells were incubated for a further 48 h to establish the cisplatin and siRNA-3 plus cisplatin groups, respectively. The single negative control group and single siRNA-3 group were incubated for a further 48 h but not treated with cisplatin. The cells of the four groups were then collected and labeled using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Apoptosis rates were determined by flow cytometry (BD Biosciences) and analyzed with FlowJo 10.0.8 software (FlowJo, LLC, Ashland, OR, USA).

Statistical analysis. Each experiment was performed in triplicate and repeated a minimum of three times, with all data presented as the mean ± standard deviation. Statistical analyses were performed using SPSS software, version 18.0. Comparisons between groups were conducted using the Student's t-test or analysis of variance, where $P<0.05$ was considered to indicate a statistically significant difference.

Results

ARID1A siRNA downregulates the protein expression levels of ARID1A. Expression levels of ARID1A were analyzed using western blotting. ES2 cells were transfected with siRNA in the presence of RNAi Max reagent for 6 h and then cultured in McCoy's 5A medium with 10% fetal bovine serum for 72 h. The results from the western blot analysis revealed that the protein expression levels of ARID1A in the cells of the siRNA-1, siRNA-2 and siRNA-3 groups were all markedly decreased and the level was the lowest in the siRNA-3 group, as compared with the non-siRNA-transfected cells (Fig. 1). Collectively, the results demonstrated that ARID1A siRNA was able to silence ARID1A expression and siRNA-3 interference was chosen for follow-up experiments.

Table I. IC_{50} of cisplatin and survival rates of ES2 ovarian clear cell carcinoma cells.

Group	IC_{50} (μM)	Survival rate[a] (%)
Negative control	52.55±3.62	52.67±2.30
Blank control	52.10±1.10	51.53±1.81
siRNA-3	77.22±5.34[b]	67.53±3.35[b]

Data are presented as mean ± standard deviation (n=3). [a]Survival rate when exposed to 50 μM cisplatin for 48 h. [b]P<0.05, siRNA-3 vs. the negative control group. siRNA, small interfering RNA.

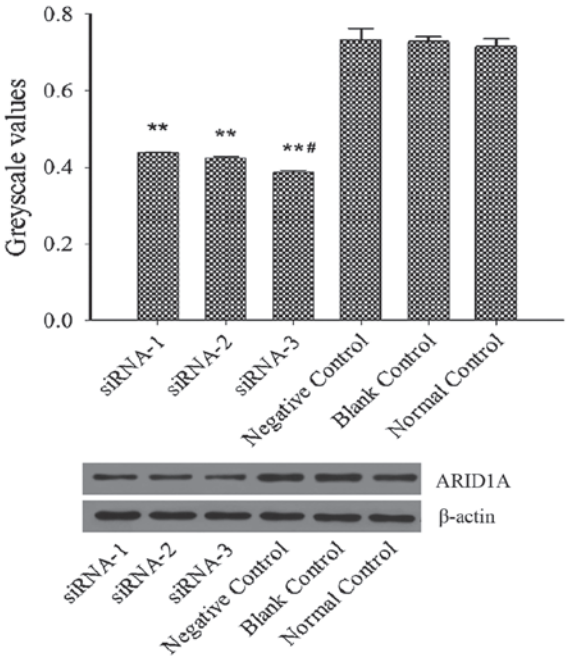

Figure 1. ARID1A siRNA downregulates the protein expression levels of ARID1A. Western blot analysis was used to quantify the expression levels of ARID1A. β-actin was used as a control for the expression levels. Data are presented as the mean + standard deviation (n=3). **P<0.01 vs. the normal control group; #P<0.05 vs. the siRNA-1 or siRNA-2 group. ARID1A, AT-rich interaction domain 1A; siRNA, small interfering RNA.

ARID1A siRNA reduces cell inhibition rate. To elucidate the effects of ARID1A gene silencing on the cisplatin sensitivity of ES2 cells, cells were exposed to different concentrations of cisplatin. As shown in Fig. 2, at the same concentration of cisplatin, the inhibitory rate of the siRNA-3 group was significantly lower than that of blank control and negative control groups (P<0.05). The IC_{50} value in the negative control group was ~52 μM (Table I), however, as shown in Table I, the IC_{50} value in the siRNA-3 group was ~77 μM which was significantly higher than that of the negative control and blank control groups (P<0.05). Then, 50 μM cisplatin was added to the 96-well culture plates following transfection and the cell survival rate of each group after 48 h incubation was calculated. The results showed that the cell survival rate of the siRNA-3 group was higher than that of the other two groups (Table I). These results indicate that ARID1A siRNA decreased the sensitivity of ES2 cells to cisplatin.

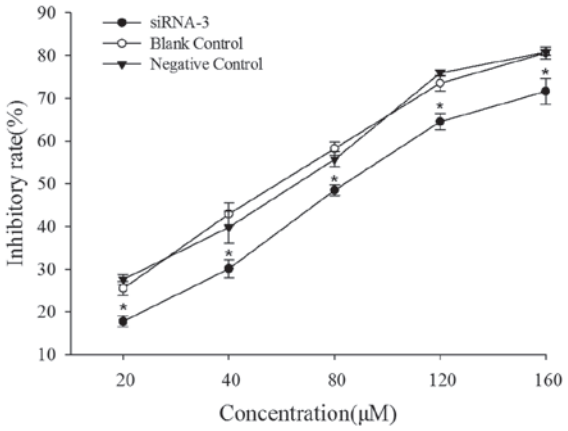

Figure 2. ARID1A siRNA reduces the cisplatin-induced cell inhibition rate. Cell Counting kit-8 assay was used to determine the inhibitory rates of different concentrations of cisplatin on ES2 cells in each group. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. the blank control or negative control group. ARID1A, AT-rich interaction domain 1A; siRNA, small interfering RNA.

ARID1A siRNA decreases cell apoptosis. ES2 cells were transfected for 6 h and then 50 μM cisplatin was added to the cells after incubation for 24 h. Apoptotic rates were determined by flow cytometry. The results (Fig. 3) indicate that the apoptotic rate of the siRNA-3 plus cisplatin group (Fig. 3C) was significantly lower than that of the cisplatin group (Fig. 3D). In addition, the apoptotic rate of the siRNA-3 group (Fig. 3B) was lower than that of the negative control group (Fig. 3A). These results demonstrate that that cell apoptotic rate decreased when the ARID1A gene was silenced.

ARID1A siRNA increases the expression levels of AKT. To elucidate the possible molecular mechanism by which ARID1A siRNA decreased the sensitivity of ES2 cells to cisplatin, the effect of ARID1A gene silencing on the AKT protein level was investigated. The results of western blot analysis (Fig. 4) demonstrated that AKT expression was upregulated in the siRNA-3 group compared with the negative control group. In addition, AKT expression in the cisplatin group was significantly decreased compared with that in the negative control group. Furthermore, AKT expression in the siRNA-3 plus cisplatin group was significantly increased compared with that in the cisplatin group. Thus, ARID1A gene silencing appeared to attenuate the suppression of AKT expression by cisplatin. These results indicate that ARID1A gene silencing decreased the sensitivity of ES2 cells to cisplatin, in part via the upregulation of AKT expression.

Discussion

OCCC is considered to be a unique clinical and pathological type of ovarian cancer due to its typical histological characteristics; the lesions coexist with ectopic endometrium, are highly resistant to chemotherapy and have a poor prognosis (2,4,12,13). Although little is known about the molecular genetic changes of tumor development, there is a widely accepted tumor hypothesis which considers that OCCC originates from precancerous lesions (such as endometriosis

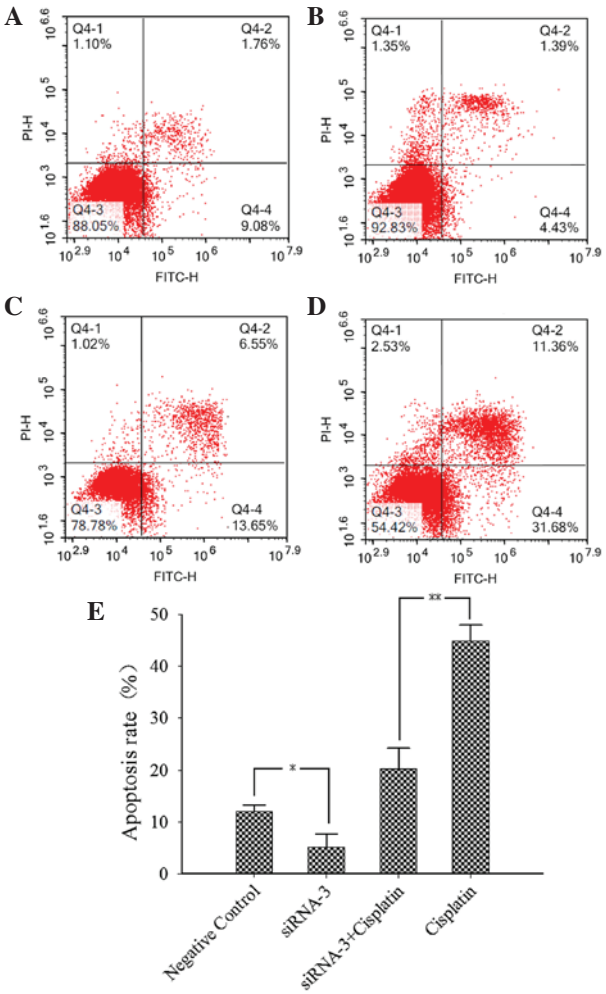

Figure 3. ARID1A siRNA decreases cell apoptosis. Flow cytometry was used to determine the cell apoptosis of the (A) negative control group, (B) siRNA-3 group, (C) siRNA-3 + cisplatin group and (D) cisplatin group. (E) Apoptosis rates in the four groups. Data are presented as the mean + standard deviation (n=3). *P<0.05; **P<0.01. ARID1A, AT-rich interaction domain 1A; siRNA, small interfering RNA.

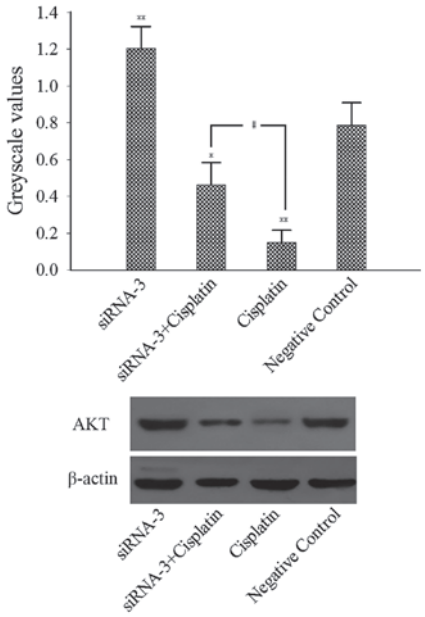

Figure 4. ARID1A siRNA increases the expression levels of AKT. Western blotting was performed to quantify the expression levels of AKT. β-actin was used as a control for the expression levels. Data are presented as the mean + standard deviation (n=3). *P<0.05 vs. the negative control group; **P<0.01 vs. the negative control group; $^{\#}$P<0.05. ARID1A, AT-rich interaction domain 1A; siRNA, small interfering RNA.

and benign clear cell adenofibroma) in the development of the corresponding atypical lesions (such as atypical endometriosis and borderline clear cell adenocarcinoma) (14,15). A previous study found that there was a loss of ARID1A expression in OCCC and adjacent to the ectopic endometrial epithelium, while ARID1A expression was retained in the cystic epithelium distant from the tumor (16). Therefore, ARID1A is considered as a potential tumor suppressor gene, and its expression is closely associated with tumor formation and prognosis (17-19). Patients with advanced OCCC are treated with cytoreductive surgery and chemotherapy with a platinum-based regimen, but the problem of drug resistance has been persistent in the clinic. The high mutation rate of ARID1A in OCCC may be the cause of the resistance to platinum and other chemotherapy drugs. In addition, a study found that after the ARID1A gene was knocked out in Jurkat leukemia cells, there was resistance to FAS-mediated apoptosis (20). The results of the present study also suggest that ARID1A gene mutation may be associated with the drug resistance of tumor cells.

The ES2 cell line is a typical OCCC cell line. In the present study, the sensitivity of ES2 cells to the chemotherapeutic drug cisplatin was detected with ARID1A gene silencing, and the results indicated that the IC_{50} of cisplatin was significantly increased compared with that of the blank control group and negative control group when ARID1A gene expression was reduced by specific siRNA. The cell survival rate of the ARID1A siRNA group was significantly higher compared with that of the negative control group following treatment with the same concentration of cisplatin (50 μM). Collectively, these results suggest that ARID1A gene silencing reduced the sensitivity of ES2 cells to cisplatin.

Further investigation into the apoptosis of ES2 cells with ARID1A gene silencing and cisplatin treatment was conducted. The results showed that the cell apoptosis rate of the ARID1A siRNA plus cisplatin group was significantly lower than that of the cisplatin group. In addition, the cell apoptosis rate of the ARID1A siRNA group was decreased compared with that of the negative control group. Therefore, these results indicate that ARID1A gene silencing decreased the apoptosis of ES2 cells and induced resistance to the killing effect of cisplatin on cells to a certain extent.

However, the drug resistance mechanism of tumor cells caused by ARID1A gene mutation is not very clear. Previous studies have reported that a variety of signaling pathways are involved in the phosphatidylinositol 3-kinases (PI3K) family, which regulates cell proliferation, differentiation and apoptosis (21). The signaling pathway constituted by PI3K type IA and its downstream molecular serine/threonine protein kinase B (AKT) is closely associated with the occurrence and development of tumors. Dysregulation of the PI3K-AKT signaling pathway exists in many human tumors, and mutations in certain components change the function of genes and thereby cause the transformation of cells. This pathway is associated with the proliferation, invasion and metastasis of

tumor cells (22,23). AKT acts on the anti-apoptotic pathway via the phosphorylation of downstream target proteins, and affects the survival of cells through acting on nuclear factor (NF)-κB and P53. AKT activates inhibitor of NF-κB (IκB) kinase by phosphorylation and results in the degradation of IκB, thereby releasing NF-κB from the cytoplasm. The released NF-κB carries out nuclear translocation, and then activates target genes to promote survival of the cells (24,25). Previous studies have shown that antisense oligonucleotide to AKT is able to inhibit the growth ability of tumor cells in soft agar, induce apoptosis and increase the sensitivity of tumor cells to chemotherapeutic agents (26). The results of the present study revealed that the AKT expression level in the cisplatin group was lower than that in the negative control group, and the AKT expression in the ARID1A siRNA plus cisplatin group was higher than that in the cisplatin group. Therefore, these results indicate that cisplatin induces cell apoptosis by decreasing AKT expression; however, ARID1A gene silencing caused AKT expression to increase, resulting in resistance to the pro-apoptotic effect of cisplatin to a certain extent, eventually leading to the ES2 cells becoming resistant to cisplatin.

In conclusion, the results of the present study demonstrated that ARID1A gene silencing reduced the sensitivity of ES2 cells to cisplatin via the regulation of AKT expression. These observations further our understanding of the association between ARID1A gene mutation and drug resistance in OCCC and may provide a novel therapeutic target for the treatment of OCCC.

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