

Knockdown of MACC1 expression increases cisplatin sensitivity in cisplatin-resistant epithelial ovarian cancer cells

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Abstract. Abnormal expression of metastasis-associated in colon cancer 1 (MACC1) was found to be closely associated with several types of malignant tumors. The present study aimed to verify the relationship between MACC1 and cisplatin resistance in ovarian cancer cells and the possible mechanisms, which was implemented by inhibition of the expression of MACC1 in cisplatin-resistant human ovarian cancer cell lines A2780/DDP and COC1/DDP. MACC1 shRNA eukaryotic plasmids and negative control plasmids were transfected into A2780/DDP and COC1/DDP cells, respectively, while A2780/DDP and COC1/DDP cells were used as blank controls. Western blotting and sqRT-PCR were used to detect the expression of MACC1 in the different cell groups. Different concentrations of cisplatin (0, 10, 20, 30, 40, 50 and 60 $\mu\text{mol/l}$) were used to treat the cell groups, respectively, and then the chemosensitivity of cisplatin and cell apoptosis were examined by MTT and flow cytometry, respectively. The activity of caspase-3 was determined by spectrophotometry. Expression levels of p-ERK1/2, permeability glycoprotein (P-gp), B-cell lymphoma 2 (Bcl-2), Bcl-X_L, Bax and Bad protein were detected in the different ovarian cancer cells by western blotting. After MACC1 knockdown, the chemosensitivity of cisplatin in the ovarian cancer cells was enhanced, and the cell growth inhibition and apoptosis rates were increased. The expression levels of Bax and Bad were upregulated, the activity of caspase-3 was increased, while the expression levels of p-ERK1/2, P-gp, Bcl-2 and Bcl-X_L were downregulated as a result of MACC1 inhibition. These results indicate that inhibition of MACC1 improves the chemosensitivity of cisplatin in epithelial ovarian cancer cells, through the regulation of the ERK1/2 signaling pathway on P-gp and its downstream apoptosis proteins.

Introduction

Epithelial ovarian cancer (EOC) is commonly detected at a late stage, and is commonly diagnosed with abdominal ascites and widespread intraperitoneal dissemination. Aggressive cytoreductive surgery, if possible, is the primary clinical treatment for advanced ovarian cancer (1,2). Platinum and paclitaxel combination chemotherapy or i.p. cisplatin based-chemotherapy is used as a first-line neoadjuvant before cytoreductive surgery or adjuvant treatment after surgery (3,4). Most patients develop platinum chemoresistance or multi-drug resistance during the chemotherapy progress, which is a major obstacle for chemotherapy in ovarian cancer patients (5). Therefore, the identification of platinum-resistance mechanisms and the exploration of chemoresistance reversing therapeutic targets for human EOC are required.

MACC1 was found to be overexpressed in colon cancer, gastric carcinoma, lung cancer, hepatocellular carcinoma and ovarian cancer, and may be used as a biomarker for the poor prognosis and the high risk of metastasis in these malignant tumors (6-10). It was found that downregulation of MACC1 by siRNA sensitized pancreatic cancer cells to gemcitabine treatment, which may be involved in the inhibition of the Ras/ERK signaling pathway (11). Another study showed that knockdown of MACC1 enhanced the apoptosis and growth inhibitory rates of human glioblastoma cells, and could increase glioblastoma cell sensitivity to cisplatin chemotherapy (12). These data indicated that, in addition to invasion and metastasis, MACC1 may also play other unknown roles in the pathological processes of cancer cells, such as chemoresistance.

Herein, we hypothesized that MACC1 may be implicated in the chemoresistance of cisplatin in ovarian cancer. To verify this hypothesis, we inhibited the expression of MACC1 in cisplatin-resistant ovarian cancer A2780/DDP and COC1/DDP cells by RNA interference. Chemosensitivity and the apoptosis rate in different ovarian cancer cells were determined following treatment with different concentrations of cisplatin. Expression levels of apoptosis-associated proteins and caspase-3 activity were assessed. The relationship between MACC1 and cisplatin resistance was investigated.

Materials and methods

Cell transfection. Human ovarian carcinoma A2780/DDP and COC1/DDP cells were purchased from the China Center

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for Type Culture Collection (Wuhan, China), and cultured in complete RPMI-1640 medium (Hyclone, Logan, UT, USA), at 37°C in 5% CO₂. Cells were harvested in the logarithmic phase of growth for all experiments as described below. Recombinant MACC1-psuper-EGFP-shRNA eukaryotic plasmids and the negative control plasmid, as constructed in our previous study (13), were transfected into the A2780/DDP and COC1/DDP cells, respectively, which was performed following the protocol of Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Stably transfected A2780/DDP and COC1/DDP cells were isolated by G418 (Sigma, St. Louis, MO, USA). Three cell groups used for the next research steps were named: blank control cells (B), negative control cells (NC) and MACC1-knockdown cells (M).

sqRT-PCR. Cell total RNA was isolated using TRIzol reagent (Invitrogen), and first strand cDNA was synthesized from 1 µg total RNA according to the protocol of the RevertAid First Strand cDNA Synthesis kit (Fermentas, EU). Primers used in the sqRT-PCR were MACC1, 5'-CCTTCGTGGTAATAATGCTTCC-3' (sense) and 5'-AGGGCTTCCATTGTATTGAGGT-3' (antisense); and β-actin, 5'-ACGCACCCCACTACAACCTC-3' (sense) and 5'-TCTCCTTAATGTCACGCACGA-3' (antisense). PCR cycling parameters (19 cycles) were: denaturation (94°C for 30 sec), annealing (56°C for 30 sec) and extension (72°C for 30 sec). Equal amounts of PCR products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining. The specific bands of the PCR products were analyzed by Image-Pro Plus 6.0 system, and β-actin was used as a control for normalization. sqRT-PCR was performed three times independently.

Western blot analysis. The antibodies used in the western blotting, following the manufacturer's protocols, included rabbit anti-human polyclonal MACC1 (Sigma), rabbit anti-human polyclonal phospho-ERK1/2 and mouse anti-human monoclonal P-gp (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human polyclonal Bcl-2, rabbit anti-human monoclonal Bcl-X_L, mouse anti-human monoclonal Bax, and rabbit anti-human polyclonal Bad (Beyotime Biotechnology, Haimen, Jiangsu, China). Total protein was extracted using RIPA lysis buffer for western blot analysis and IP (Beyotime Biotechnology), and the protein concentration was determined using Bradford assay. Equal amounts of protein (30 µg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. The detection of hybridized protein was performed by enhanced chemiluminescence kit (Zhongshan Goldenbridge Biotechnology, Peking, China), and β-actin was used as a control for normalization. The relative values of the specific bands were analyzed by Image-Pro Plus 6.0 system three times independently.

MTT assay. Cells were planted (1x10⁴ cells/well) into 96-well plates, and 100 µl complete RPMI-1640 medium containing 10% FBS was added into each well. Three duplicate wells were set up for each group. The cells were cultured for 24 h, and then medium containing cisplatin at 0, 10, 20, 30, 40, 50, and 60 µmol/l, respectively was added. The cells were incubated for 48 h, and the previous medium was replaced by 100 µl complete RPMI-1640 medium in each well. After 24 h,

20 µl MTT reagent (5 mg/ml; Sigma) was added into each well. Cells were then incubated for another 4 h and then the former medium was aspirated and 150 µl DMSO was added. The absorbance of the sample was measured by a microplate spectrophotometer (Thermo Spectronic, Madison, WI, USA) at 492 nm. All experiments were conducted in triplicate. Cell growth inhibition rate and the half inhibitory concentration (IC₅₀) value of cisplatin (LOGIT assay) were calculated. Cell growth inhibition rate = (1 - sample OD/control OD) x 100%.

Flow cytometric analysis. Every cell group was captured for 48 h in medium containing cisplatin at 0, 10, 20, 30, 40, 50 and 60 µmol/l, respectively. The cells were incubated for another 24 h with complete RPMI-1640 medium. Approximately 1x10⁶ cells were treated into a single-cell suspension with PBS solution, and were prepared following the manufacturer's protocol in the Annexin V-FITC Apoptosis Detection kit (Beyotime Biotechnology). Then, rates of apoptosis were analyzed with FACScan system (BD Biosciences, San Jose, CA, USA).

Caspase-3 activity assay. Every cell group was cultured for 48 h in medium containing cisplatin at 0, 10, 20, 30, 40, 50 and 60 µmol/l, respectively, and then incubated for another 24 h with completed RPMI-1640 medium. Approximately 1x10⁶ cells were collected, total protein was extracted using RIPA lysis buffer for western blot analysis and IP (Beyotime Biotechnology), and the protein concentration was controlled using Bradford assay (between 1 to 3 mg/ml as requested). Equal volumes of protein (50 µl) were prepared following the manufacturer's protocol in the Caspase-3 Activity Detection kit (Beyotime Biotechnology). The absorbance values were measured by a microplate spectrophotometer (Thermo Spectronic) at 405 nm. The pyrolysis liquid without the cell samples was used as a blank control. The value of ΔA405 (absorbance value of sample at 405 nm minus absorbance value of blank control at 405 nm) represents the activity of caspase-3. All experiments were performed in triplicate.

Statistical analysis. Average values are expressed as mean ± standard deviation (SD). Measurement data were analyzed by one-way ANOVA, non-parametric test and Bonferroni test using SPSS 17.0 software package. A difference was considered significant at P<0.05.

Results

MACC1 mRNA and protein expression is altered after RNA interference. As a result of MACC1 knockdown, significant downregulation of MACC1 was observed in the A2780/DDP-M and COC1/DDP-M cells (P<0.05). No differences were noted between the A2780/DDP-B and A2780/DDP-NC cells, and between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 1).

Suppression of cell proliferation after MACC1 knockdown. After incubation with different concentrations of cisplatin, cell growth inhibition rates were obviously increased in the A2780/DDP-M and COC1/DDP-M cells ($\chi^2=9.029$, P=0.011; $\chi^2=6.226$, P=0.044). With higher concentrations of cisplatin,

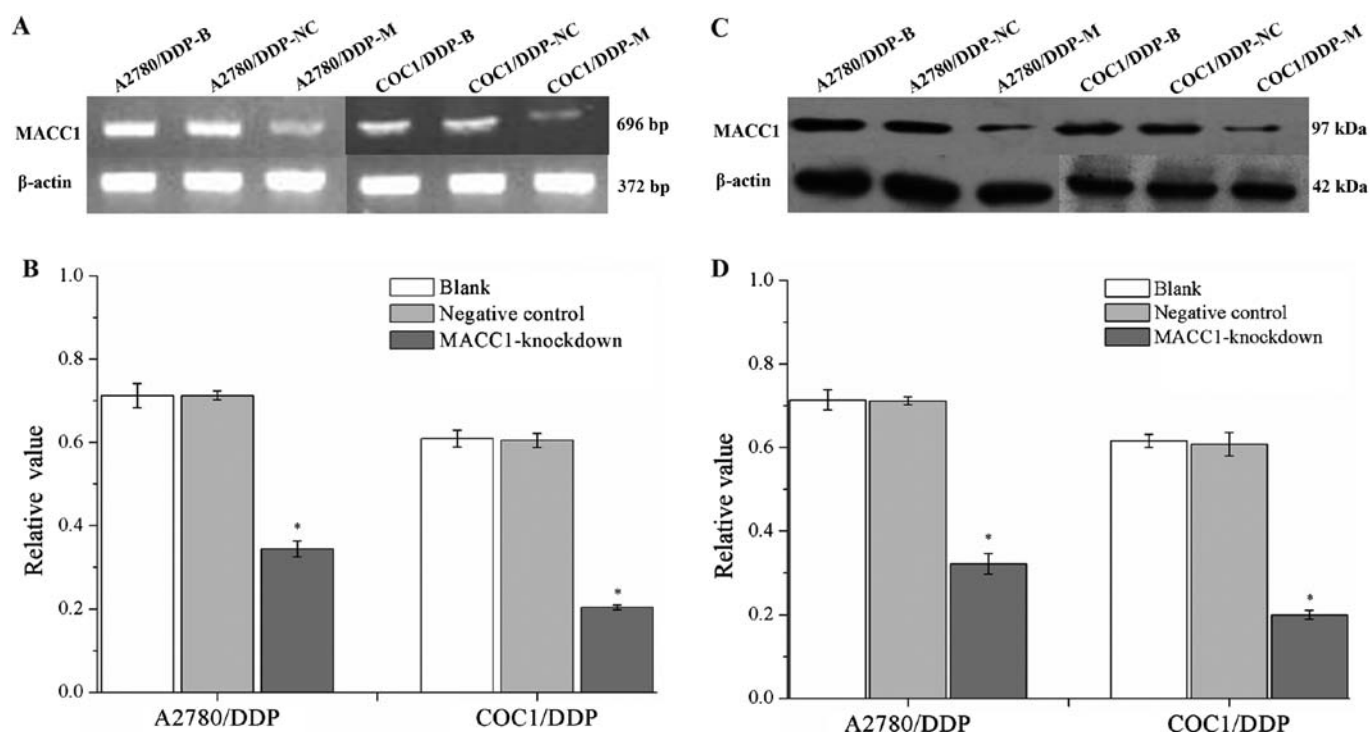


Figure 1. Expression levels of MACC1 mRNA and protein as detected by sqRT-PCR and western blotting in the different cell groups. (A) Expression of MACC1 mRNA as detected by sqRT-PCR in the different cell groups. (B) Histogram shows the relative values of MACC1 mRNA as measured by sqRT-PCR. Each bar represents the mean \pm SD. * P <0.05 compared with the blank and negative control groups. (C) Expression of MACC1 protein as detected by western blotting in the different cell groups. (D) Histogram shows the relative values of MACC1 protein as measured by western blotting. Each bar represents the mean \pm SD. * P <0.05 compared with the blank and negative control groups.

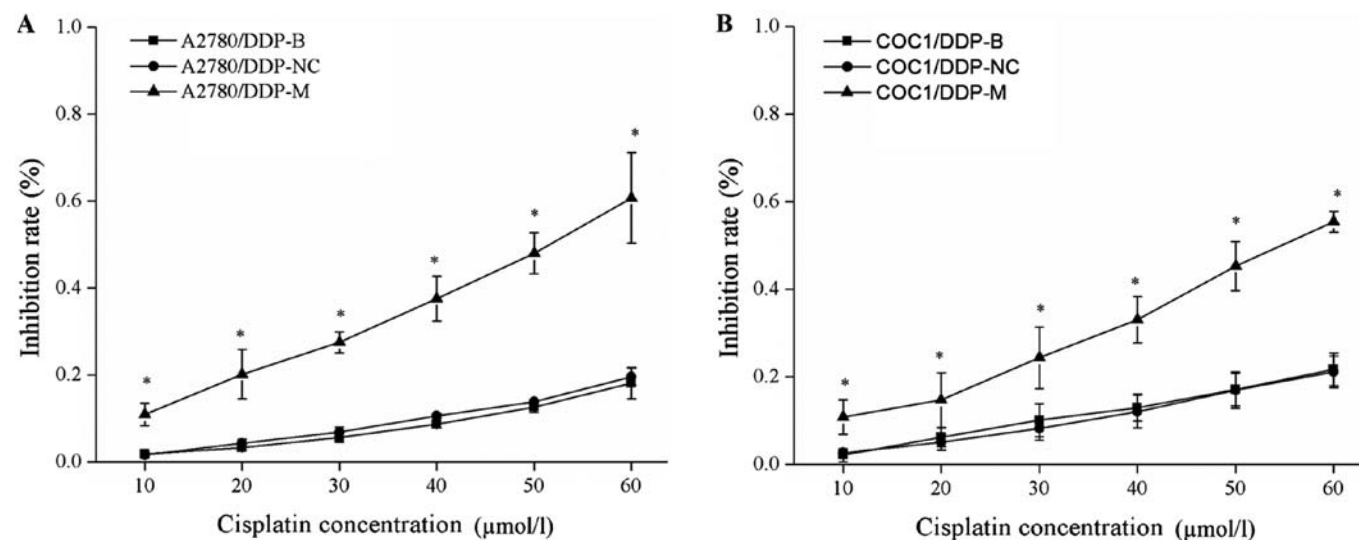


Figure 2. Cell growth inhibition rates as measured by MTT assay in the different cell groups following treatment with different concentrations of cisplatin, respectively. (A) Line graph represents the cell growth inhibition rates in the different A2780/DDP cells. Each bar represents the mean \pm SD. * P <0.05 compared with the blank and negative control groups. (B) Line graph represents the cell growth inhibition rates in different COC1/DDP cells. Each bar represents the mean \pm SD. * P <0.05 compared with the blank and negative control groups.

increased rates of cell growth inhibition were noted (P <0.05). No differences were found between the A2780/DDP-B and A2780/DDP-NC cells, between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 2).

In addition, the IC_{50} values of A2780/DDP-M and COC1/DDP-M cells for cisplatin were obviously decreased compared with the control cells ($F=22.760$, $P=0.002$;

$\chi^2=6.489$, $P=0.039$). No differences were noted between the A2780/DDP-B and A2780/DDP-NC cells, and between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 3).

Cell apoptosis is induced by RNA interference. After incubation with different concentrations of cisplatin, the cell apoptosis rates were markedly increased in the A2780/DDP-M

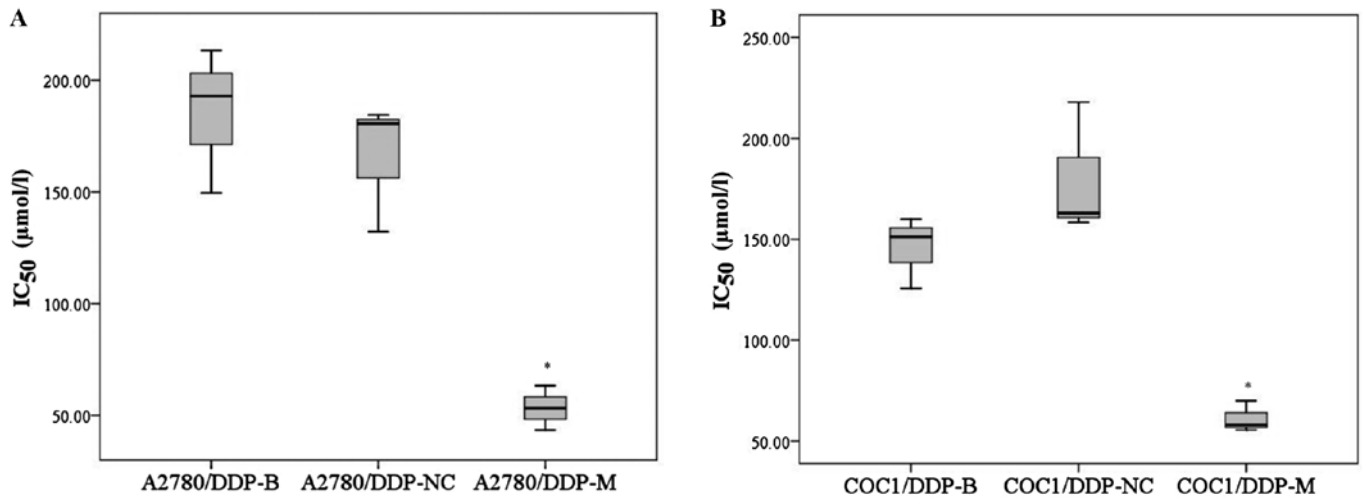


Figure 3. Cisplatin IC₅₀ values as determined by MTT assay in the different cell groups following treatment with different concentrations of cisplatin, respectively. (A) Box plots represent the IC₅₀ values in the different A2780/DDP cells. Each bar represents the mean ± SD. *P<0.05 compared with the blank and negative control groups. (B) Box plots represent the IC₅₀ values in the different COC1/DDP cells. Each bar represents the mean ± SD. *P<0.05 compared with the blank and negative control groups.

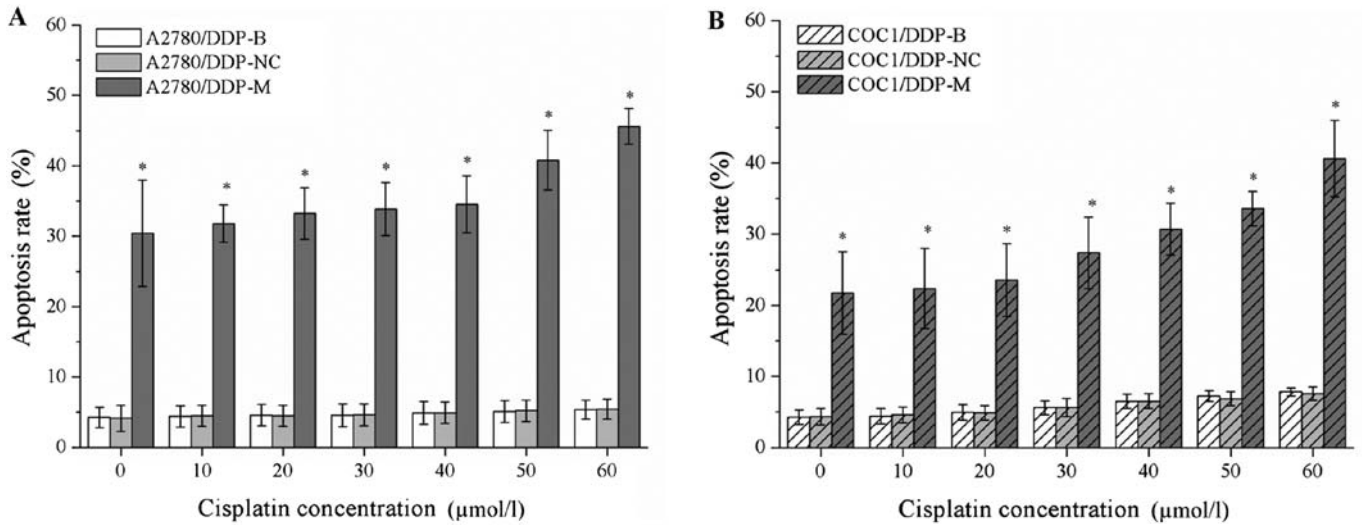


Figure 4. Cell apoptosis rates as determined by flow cytometry in the different cell groups following treatment with different concentrations of cisplatin, respectively. (A) Histogram represents apoptosis rates in the different A2780/DDP cells. Each bar represents the mean ± SD. *P<0.05 compared with the blank and negative control groups. (B) Histogram represents the apoptosis rates in the different COC1/DDP cells. Each bar represents the mean ± SD. *P<0.05 compared with the blank and negative control groups.

and COC1/DDP-M cells ($\chi^2=41.375$, $P=0.000$; $\chi^2=41.362$, $P=0.000$). For a higher concentration of cisplatin, greater rates of cell apoptosis were observed ($P<0.05$). No differences were noted between the A2780/DDP-B and A2780/DDP-NC cells, and between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 4).

Expression of p-ERK1/2, P-gp, Bcl-2, Bcl-X_L, Bax and Bad in the different cell groups. After MACC1 knockdown, obvious downregulation of p-ERK1/2, P-gp, Bcl-2 and Bcl-X_L was detected in the A2780/DDP-M and COC1/DDP-M cells ($P<0.05$). In contrast, upregulation of Bax and Bad was found in the A2780/DDP-M and COC1/DDP-M cells ($P<0.05$). No differences in these proteins were noted between the A2780/DDP-B and A2780/DDP-NC cells, and between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 5).

Caspase-3 activity is increased by RNA interference. After incubation with different concentrations of cisplatin, the values of ΔA_{405} were markedly increased in the A2780/DDP-M and COC1/DDP-M cells ($\chi^2=41.345$, $P=0.000$; $\chi^2=41.349$, $P=0.000$). At a higher concentration of cisplatin, a greater value for ΔA_{405} was observed ($P<0.05$). These data indicate that the activity of caspase-3 was increased in the A2780/DDP-M and COC1/DDP-M cells. No differences were noted between the A2780/DDP-B and A2780/DDP-NC cells, and between the COC1/DDP-B and COC1/DDP-NC cells (Fig. 6).

Discussion

Platinum compounds are the mainstay of chemotherapy for ovarian cancer, particularly for advanced patients. Cisplatin, a conventional chemotherapy drug, has long-term clinical

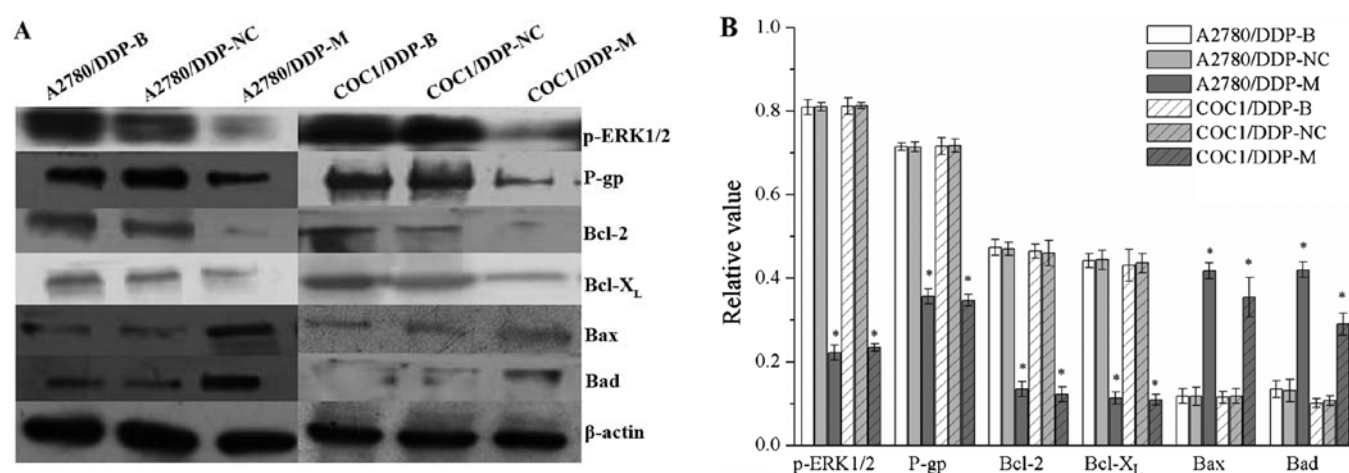


Figure 5. Expression levels of p-ERK1/2, P-gp, Bcl-2, Bcl-X_L, Bax and Bad proteins following MACC1 knockdown as detected by western blotting. (A) Expression levels of p-ERK1/2, P-gp, Bcl-2, Bcl-X_L, Bax and Bad proteins as detected by western blotting in the different cell groups. (B) Histogram shows the relative values of p-ERK1/2, P-gp, Bcl-2, Bcl-X_L, Bax and Bad proteins determined by western blotting. Each bar represents the mean \pm SD. *P<0.05 compared with the blank and negative control groups.

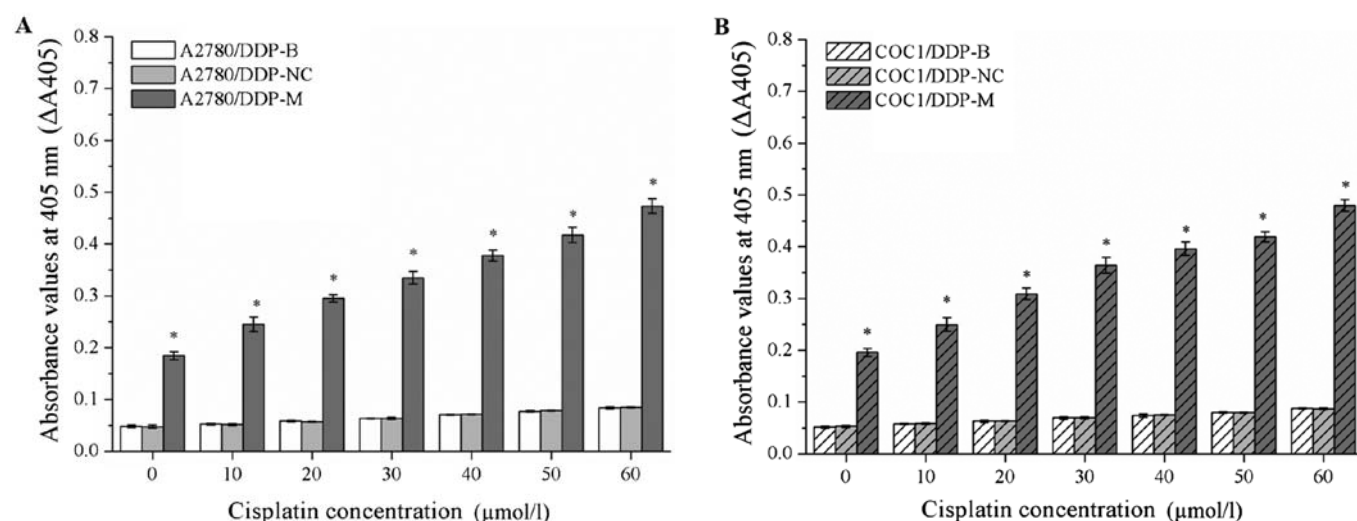


Figure 6. The values of $\Delta A405$ as determined by a spectrophotometer in the different cell groups following treatment with the different concentrations of cisplatin, respectively. The value of $\Delta A405$ (absorbance value of the sample at 405 nm minus the absorbance value of the blank control at 405 nm) represents the activity of caspase-3. (A) Histogram represents $\Delta A405$ values in the different A2780/DDP cells. Each bar represents the mean \pm SD. *P<0.05 compared with the blank and negative control groups. (B) Histogram represents $\Delta A405$ values in the different COC1/DDP cells. Each bar represents the mean \pm SD. *P<0.05 compared with the blank and negative control groups.

application in ovarian cancer (14). Unfortunately, intrinsic or acquired platinum resistance remains a critical obstacle for chemotherapy treatment of epithelial ovarian cancer. After tumor cytoreductive surgery, only 70-80% of ovarian cancer cases are responsive to platinum combined chemotherapy (15).

MACC1 has been found to be associated with invasion and metastasis in numerous types of malignant tumors. Our previous studies showed that MACC1 mRNA and protein were overexpressed in ovarian cancer tissues and cells. Inhibition of MACC1 by RNA interference suppressed the invasion and metastatic potential of ovarian carcinoma cells *in vitro* and *in vivo*, and the antitumor effects of MACC1 knockdown may involve the inhibition of the MEK/ERK1/2 pathways (10,13).

Recently, it has been reported that small interfering RNA targeting MACC1 attenuates cisplatin resistance in tongue

squamous cell carcinoma cells (16). However, another research team claimed that downregulation of MACC1 expression by RNA interference technology had no effect on cisplatin resistance in salivary adenoid cystic carcinoma cells (17). The present results showed that MACC1 was obviously downregulated in the A2780/DDP and COC1/DDP cells by RNA interference. After MACC1 knockdown, cell growth was repressed, IC₅₀ values of cisplatin were decreased, and the cell apoptosis rate was increased in the A2780/DDP and COC1/DDP cells following treatment with different concentrations of cisplatin. These data indicate that inhibition of MACC1 improves the cisplatin sensitivity of ovarian cancer cisplatin-resistant cells, and MACC1 may be involved in the chemoresistance of cisplatin in ovarian cancer.

The MAPK-ERK1/2 pathway has been implicated in cell survival, anti-apoptosis, invasion, metastasis, angiogenesis

and chemoresistance of malignancies, including ovarian carcinoma (18-21). P-glycoprotein (P-gp), also known as MDR1 or the ATP-binding cassette subfamily B member 1, is involved in the drug resistance of tumor cells. P-gp is important for transporting drugs across the cell membrane to pump drugs out of the cells and to decrease intracellular drug concentrations (22). Inhibition of MDR1 or P-gp expression can enhance drug chemosensitivity in malignant tumor cells, including ovarian cancer (23-26). Chemotherapy drugs play therapeutic roles in cancer through different mechanisms, mainly by inhibition of cell growth and induction of cell apoptosis. The Bcl-2 gene family plays key roles in cell apoptosis and cancer therapy (27). Bcl-2 and Bcl-X_L are important anti-apoptotic proteins; Bax and Bad are pro-apoptotic proteins. Overexpression of Bcl-2 leads to drug-resistance to cisplatin in ovarian tumor cells, and Bcl-2 inhibition by gene silencing induces cell apoptosis and reverses drug resistance (28,29). Caspase-3 is the most important apoptotic protease cascade protein in the caspase family, which can implement programmed cell death under the regulation of Bcl-2 family proteins (30,31).

Following MACC1 knockdown, expression levels of p-ERK1/2, P-gp, Bcl-2 and Bcl-X_L protein were obviously decreased, while expression levels of Bax and Bad were significantly increased in this study. Furthermore, caspase-3 activity was markedly increased in the MACC1-knockdown ovarian cancer cells. These results elucidate the possible mechanisms of MACC1 involved in the cisplatin-resistance of ovarian cancer cells. These mechanisms may include the regulation of P-gp expression through the ERK1/2 signaling pathway, which affects the expression of pro-apoptotic and anti-apoptotic proteins, and the activity of caspase-3 downstream.

In conclusion, the present study demonstrated that suppression of MACC1 improves the chemosensitivity of cisplatin in epithelial ovarian cancer cells, which may be through regulation of the ERK1/2 signaling pathway and P-gp and its downstream apoptotic proteins.

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