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Downregulation of *MACC1* expression enhances cisplatin sensitivity in SKOV-3/DDP cells

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ABSTRACT. The aim of this study was to investigate the correlation between *MACC1* expression and resistance to cisplatin (DDP) in DDP-resistant human epithelial ovarian cancer SKOV-3 cells (SKOV-3/DDP). *MACC1* mRNA and protein expression levels in SKOV-3 and SKOV-3/DDP cells were detected by reverse transcriptase polymerase chain reaction and western blot. The SKOV-3/DDP cells were divided into 5 groups: control, shVect (transfected with p-super-EGFP-1 plasmid), pshMACC1 (transfected with psuper-EGFP-shMACC1 plasmid), PD (pretreated with 20 μM PD98059), and combined (transfected with psuper-EGFP-shMACC1 plasmid and pretreated with 20 μM PD98059) groups. Cisplatin sensitivity and cell apoptosis in SKOV-3/DDP cells were assayed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and flow cytometry. ERK1/2 and p-ERK1/2 expression was determined by western blot. *MACC1* mRNA and protein expression levels in SKOV-3/DDP cells were 2.66 ± 0.54 and 1.95 ± 0.45 times those seen in SKOV-3 cells ($P < 0.05$). Cisplatin sensitivity of pshMACC1 group was much higher than that in the control and shVect groups. Cisplatin-induced cell apoptosis rates increased significantly in the pshMACC1, PD, and combined groups, compared to the control and shVect groups. Moreover, the apoptosis rate

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was the highest in the combined group among the 5 groups (IC_{50} = 20.836 ± 0.629 µM). p-ERK1/2 expression decreased significantly in the pshMACC1, PD, and combined groups (this decrease was the most obvious in the combined group). In conclusion, downregulation of *MACC1* expression could enhance cisplatin sensitivity and decrease drug resistance in SKOV-3/DDP cells.

Key words: MACC1; SKOV-3/DDP; ERK; Cisplatin sensitivity

INTRODUCTION

Ovarian cancer is one of the most common gynecological malignancies, with a high rate of morbidity and mortality. The lack of methods available for early diagnosis has led to a majority of women being diagnosed during an advanced stage of the malignancy. Despite the improvement in combined chemotherapy, the recurrence rate of ovarian cancer remains as high as 85%, while the 5-year survival rate remains less than 30% (Siegel et al., 2012). More than 70% of the patients are diagnosed during the later stages of cancer, and approximately 70% of the patients relapse within 2 years (Hennessy et al., 2009; Vergote et al., 2010). Surgery, combined with chemotherapy, is the main therapeutic strategy for ovarian cancer; however, the development of resistance towards chemotherapeutic drugs plays a majorrole in constraining the rate of curing of ovarian cancer, with the prognosis largely depending on the response of the patient to chemotherapy. The 5-year survival rate of ovarian cancer ranges from 20 to 30% because of primary or secondary drug resistance.

Metastasis-associated in colon cancer-1 (MACC1) was first discovered by Stein and was found to be closely related to colon cancer metastasis (Stein et al., 2009). MACC1 is a key regulator of hepatocyte growth factor (HGF)/c-Met signaling, which promotes colon cancer cell proliferation, invasion, and metastasis by enhancing c-Met transcription (Stein et al., 2010). Recent studies have shown that MACC1 is over-expressed in ovarian cancer (Zhang et al., 2011a), lung cancer (Shimokawa et al., 2011), gastric carcinoma (Shirahata et al., 2010), and hepatocellular carcinoma (Shirahata et al., 2011). Therefore, the over-expression of *MACC1* is related to carcinoma progression and the prognosis of patients with carcinomas.

To date, the correlation between *MACC1* expression and chemotherapy resistance in ovarian cancer remains largely unknown. In this study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and flow cytometry (FCM)-based assays were used to investigate the correlation between *MACC1* expression and cisplatin resistance in cisplatin (DDP)-resistant human epithelial ovarian cancer SKOV-3 cells (SKOV-3/DDP).

MATERIAL AND METHODS

Materials

Fussed constructs of psuper-EGFP-shMACC1 and psuper-EGFP-neo were generated at the First Affiliated Hospital of Zhengzhou University. psuper-EGFP-1 was provided by Dr. Qinxian Zhang, of the Basic Medical College of Zhengzhou University, China. The SKOV-3/DDP cell line was purchased from the Cancer Institute & Hospital, Chinese Academy of Medical Sciences (Beijing, China). The anti-MACC1 antibody was purchased from Abcam (Cambridge, UK), and the

goat anti-rabbit IgG was obtained from LifeSpan BioSciences (Seattle, WA, USA). Anti-β-actin and anti-caspase-3 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-ERK1/2 and anti-p-ERK1/2 antibodies were obtained from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). PD98059 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines and cell culture

SKOV-3/DDP and SKOV3 cell lines were cultured in RPMI1640 supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in a 5% CO_2 environment. SKOV-3/DDP cells were treated with 5 μM cisplatin to maintain drug resistance upon passaging.

Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR

The cells were plated on 6-well plates, and total RNA was extracted from the treated cells by using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer instructions. cDNA was synthesized using the RT reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). The primers were synthesized by Sangon Biotechnology (Shanghai, China), and the primer sequences are as follows: MACC1: forward, 5'-CCTTCGTGGTAATAATGC TTCC-3'; reverse, 5'-AGGGCTTCCATTGTATTGAGGT-3' and GAPDH: forward, 5'-TGAACGGGA AGCTCACTGG-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. The samples were amplified using the following reaction conditions: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C for 30 s, a final extension at 72°C for 2 min, and termination at 4°C. The PCR products were analyzed by agarose gel electrophoresis (2% gel) and visualized using Quantity Onegel imaging system (Bio-Rad, Hercules, CA, USA). The results were analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Western blot analysis

The cells were lysed with radioimmunoprecipitation assay lysis buffer containing a protease inhibitor cocktail (Sangon Biotechnology, Shanghai, China). Protein concentrations were determined using a standard bicinchoninic acid (BCA) protein assay kit (Sangon Biotechnology, Shanghai, China). Equal amounts of lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Hybond-C nitrocellulose membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The membranes were blocked for 1 h at 25°C and incubated overnight at 4°C in TBST buffer (5% milk) containing anti-MACC1 or anti-β-actin antibodies. The membranes were then washed twice with TBST buffer and incubated for 2 h at 25°C in TBST buffer (supplemented with 5% milk to block non-specific binding) containing goat anti-rabbit IgG. The blots were visualized by incubating with standard enhanced chemiluminescenceagents (Bio-Rad, Hercules, CA, USA). The bands were quantified by the Quantity One software and statistically analyzed. The data was calculated from three repeats of the experiments.

MTT assay

The cell proliferation and drug resistance capacities were analyzed by the MTT assay.

Briefly, the cells were transfected with psuper-EGFP-shMACC1 and psuper-EGFP-neoplasmids and plated on 96-well plates. The treated cells were washed twice with PBS and incubated with serum-free DMEM containing 0.5 mg/mL MTT for 4 h. The cells were then washed with PBS, and the formazan blue formed by the living cells was dissolved in dimethyl sulfoxide (DMSO). Finally, the optical density was measured at 490 nm.

Flow cytometry

The cells were transfected with the respective plasmids for 24 h, subsequently plated on 6-well plates, and divided into three groups: the control group, shVect group (transfected with the p-super-EGFP-1 plasmid), and the pshMACC1 group (transfected with the psuper-EGFP-shMACC1 plasmid). The cells were then treated with 20 μM cisplatin or negative control DMSO for 48 h. The extracted cells were washed with PBS on ice and centrifuged at 2000 *g* for 5 min at 4°C. The cells were re-suspended in 500 μL binding buffer, 5 μL Annexin V-fluorescein isothiocyanate (FITC), and 5 μL propidiumiodide, and incubated at 25°C for 15 min in the dark. Cell apoptosis was determined using the LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). All experiments were repeated thrice.

Statistical analysis

The data was analyzed on the SPSS v.13.0 software platform (IBM, Armonk, NY, USA), and were expressed as mean ± standard deviation. Data between multiple groups and two groups were analyzed by one-way analysis of variance (ANOVA) or unpaired Student's *t*-test, respectively. $P < 0.05$ was considered to bestatistically significant.

RESULTS

MACC1 mRNA and protein expression in SKOV-3 and SKOV-3/DDP cell lines

As shown in Figure 1, *MACC1* mRNA level in SKOV-3/DDP cells was approximately 2.5 (2.66 ± 0.54) times that in SKOV-3 cells ($P < 0.05$). *MACC1* expression in SKOV-3/DDP cells was (1.95 ± 0.45) times that in SKOV-3 cells, with a significant difference (Figure 2). The effect of *MACC1* on SKOV-3/DDP cell proliferation was analyzed by transfecting psuper-EGFP-shMACC1 (shMACC1 group) and the psuper-EGFP-neo (shVector group) plasmids for *MACC1* knockdown and as the negative control, respectively. We observed no significant difference in *MACC1* mRNA expression between the shVector group and control group (Figure 3). We observed a significant decrease in the *MACC1* mRNA level in the shMACC1 group compared to those in the control and shVector groups, which indicated that psuper-EGFP-shMACC1 plasmid transfection efficiently knocked down *MACC1* ($P < 0.05$). In addition, MACC1 expression also decreased significantly in the shMACC1 group compared to the control and shVector groups (Figure 4). We observed no significant difference in the rate of cell proliferation between the control and shVector groups (Figure 5). However, the rate of cell proliferation rate decreased significantly in the shMACC1 group compared to those in theshVector and control groups ($P < 0.05$).

Effects of MACC1 on SKOV-3/DDP cisplatin resistance

We also investigated the effects of MACC1 on SKOV-3/DDP cisplatin resistance. The

cells in the three groups were treated with different concentrations of cisplatin for 24, 48, and 72 h, respectively. We observed no significant difference in the cell survival rate among the control, shVector, and shMACC1 groups after cisplatin treatment for 24 h (Figure 6). Cisplatin treatment for 48 h led to a significant decrease in the cell survival rate of the shMACC1 group; the decrease was the most obvious at 20 μM. Additionally, the survival rates of cells in the shMACC1 group decreased significantly when treated with different concentrations of cisplatin, compared to those of the cells of the control and shVector groups treated for 72 h. Our results showed that the downregulation of MACC1 could result in a decrease in SKOV-3/DDP cisplatin resistance.

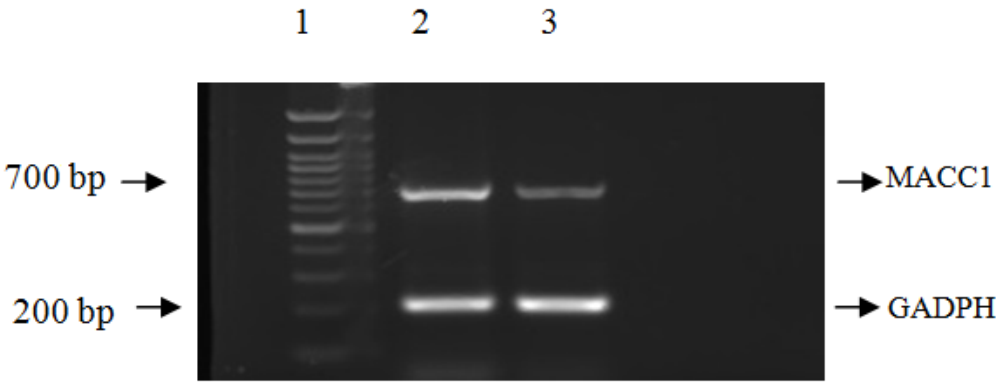

Figure 1. *MACC1* mRNA transcription in SKOV-3/DDP and SKOV-3 cell lines. Number 1, 2, and 3 represent DNA marker, SKOV-3/DDP, and SKOV-3 cell lines respectively.

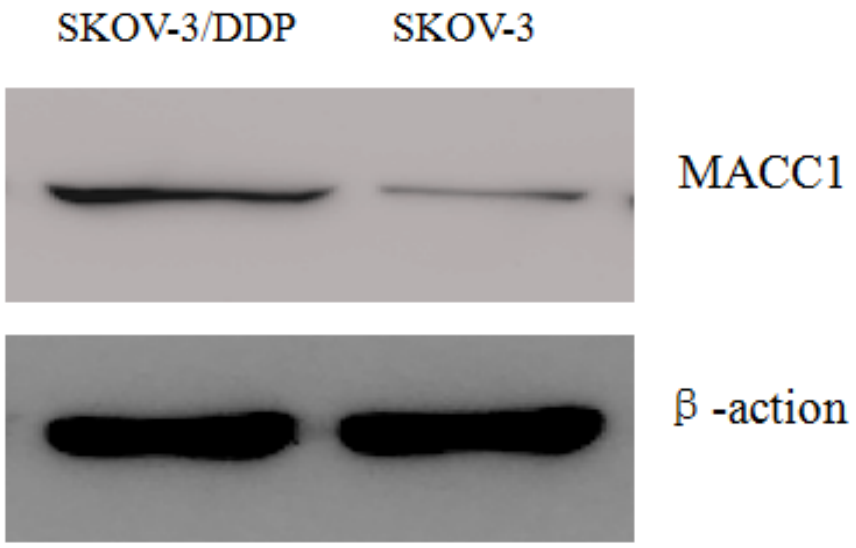

Figure 2. MACC1 protein expression in SKOV-3/DDP and SKOV-3 cell lines.

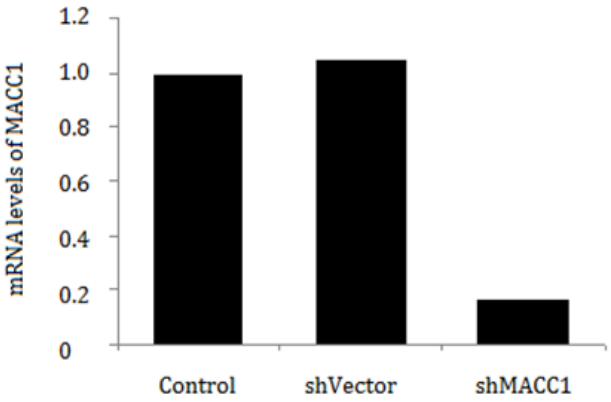

Figure 3. *MACC1* mRNA expression after transfection.

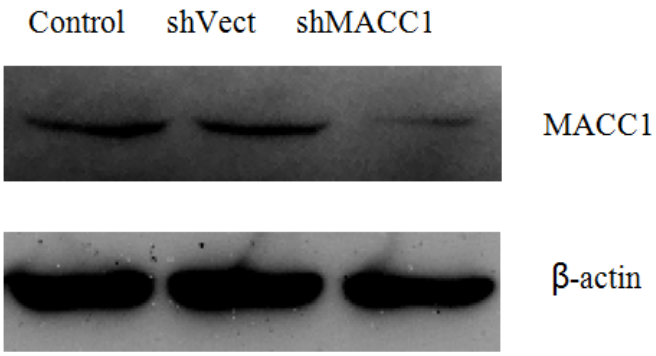

Figure 4. *MACC1* knock down in SKOV-3/DDP cells.

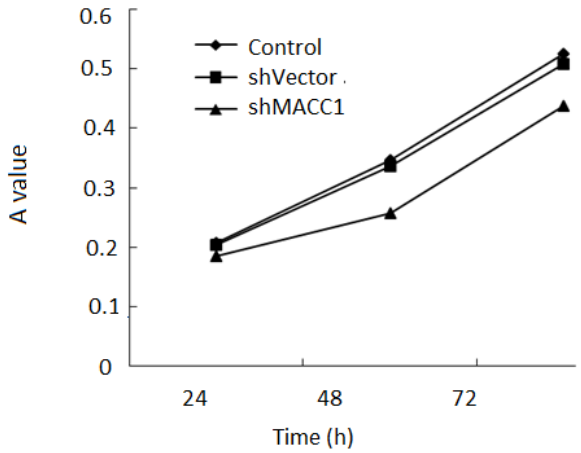

Figure 5. The growth curve of 3 ovarian cancer cell lines.

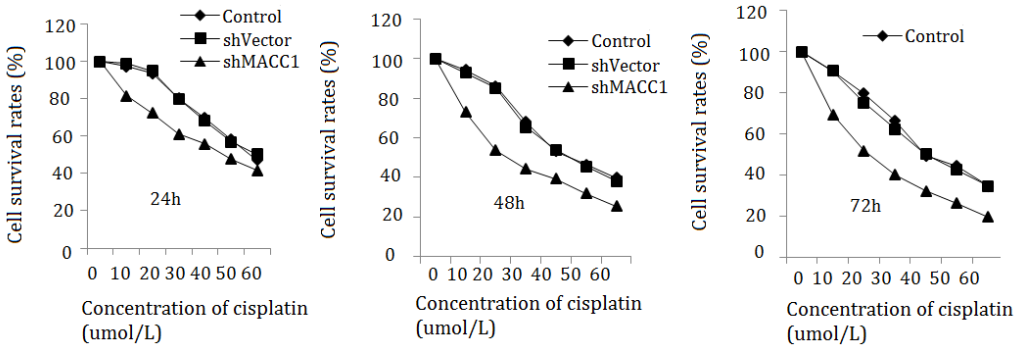

Figure 6. Effects of *MACC1* on SKOV-3/DDP cisplatin resistance.

Effects of MACC1 on cisplatin-induced cell apoptosis in SKOV-3/DDP cells

The cells were treated with 20 μM cisplatin for 48 h to investigate the effects of MACC1 on cisplatin-induced cell apoptosis in SKOV-3/DDP cells. We first determined the apoptosis rates of the three groups prior to cisplatin treatment. Early apoptosis rates in the control and shVector groups were 3.63 ± 0.31% and 4.23 ± 0.31%, respectively (Figure 7). However, the early apoptosis rate of the shMACC1 group was much higher (13.7 ± 0.60%). Cisplatin treatment for 48 h led to a decrease in the survival rates of the cells in the control and shVector groupsto 74.67 ± 0.90% and 71.9 ± 2.56%, respectively. On the other hand, the cell survival rate in the shMACC1 group was 58.77 ± 3.01%, with a significant difference. Our results showed that the down-regulation of MACC1 could increase cisplatin-induced cell apoptosis in SKOV-3/DDP cells.

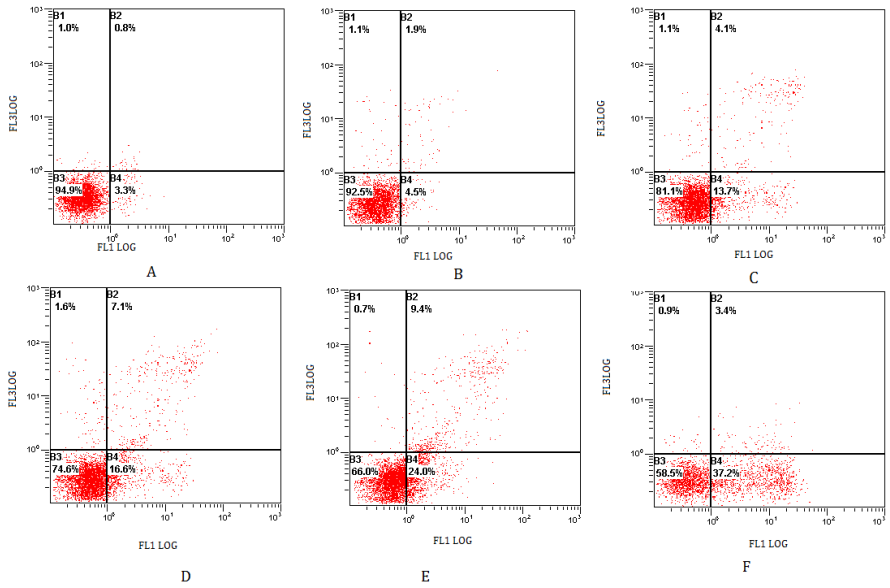

Figure 7. Effects of *MACC1* on cisplatin-induced cell apoptosis in SKOV-3/DDP cells. **A.** cell control group; **B.** empty plasmid group; **C.** pshMACC1 group; **D.** cell control group treated with cisplatin; **E.** empty plasmid group treated with cisplatin; **F.** pshMACC1 group treated with cisplatin.

MACC1 siRNA and PD98059 enhanced cisplatin sensitivity in a coordinated manner

As shown in Figure 8, the rates of inhibition of cell growth in the phMACC1, PD, and combined groups were much higher than those in the control and shVect groups, when treated with different concentrations of cisplatin. The IC50 of cisplatin in the control, shVect, pshMACC1, PD, and combined groups were 47.501 ± 0.401 μM, 47.089 ± 0.451 μM, 26.094 ± 0.911 μM, 37.998±1.024 μM and 20.836 ± 0.629 μM, respectively. Our results indicated that MACC1 downregulation could enhance cisplatin sensitivity. The inhibition rate in the combined group was much higher than that in the pshMACC1 and PD groups.

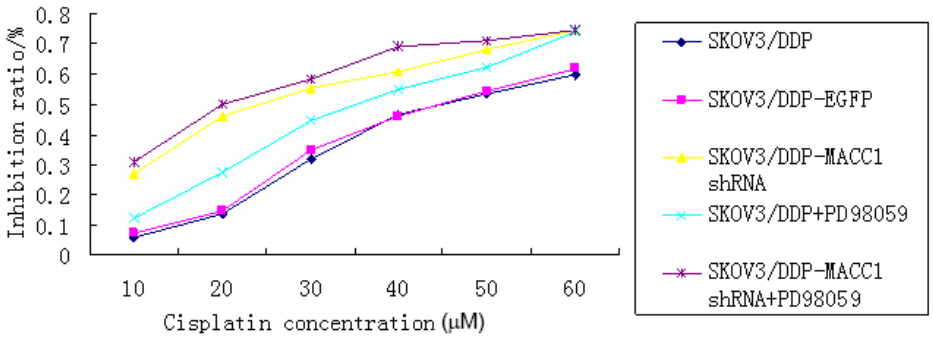

Figure 8. Determination of cisplatin IC_{50} in 5 groups.

MACC1 siRNA and PD98059 increase cisplatin-induced cell apoptosis in a coordinated manner

The rate of cell apoptosis in the 5 groups increased significantly after treatment with cisplatin ($P < 0.05$; Figure 9, Table 1). Moreover, the cell apoptosis rates in the pshMACC1, PD, and combined groups were much higher than those in the control and shVect groups ($P < 0.05$). The cell apoptosis rate was the highest in the combined group among the 5 groups.

Effects of MACC1 on p-ERK1/2 in SKOV-3/DDP cells

We also investigated the effects of MACC1 on ERK signaling. As shown in Table 2, there was no significant difference in the p-ERK1/2 level between the control and shVect groups. The downregulation of MACC1 efficiently decreased p-ERK1/2 level; the ERK inhibitor PD98059 also decreased the expression of p-ERK1/2 in this groups. Moreover, the p-ERK1/2 level was much lower in the combined group than in the shMACC1 and PD groups.

DISCUSSION

Ovarian cancer is one of the most common causes of cancer-related mortality in females. Drug resistance is a key factor influencing the prognosis of patients with ovarian cancer. Drug resistance is related to decreased uptake of drugs, alterations in the cell cycle, enhanced DNA repair activity, and defective apoptosis, among others. Investigations into the molecular mechanism

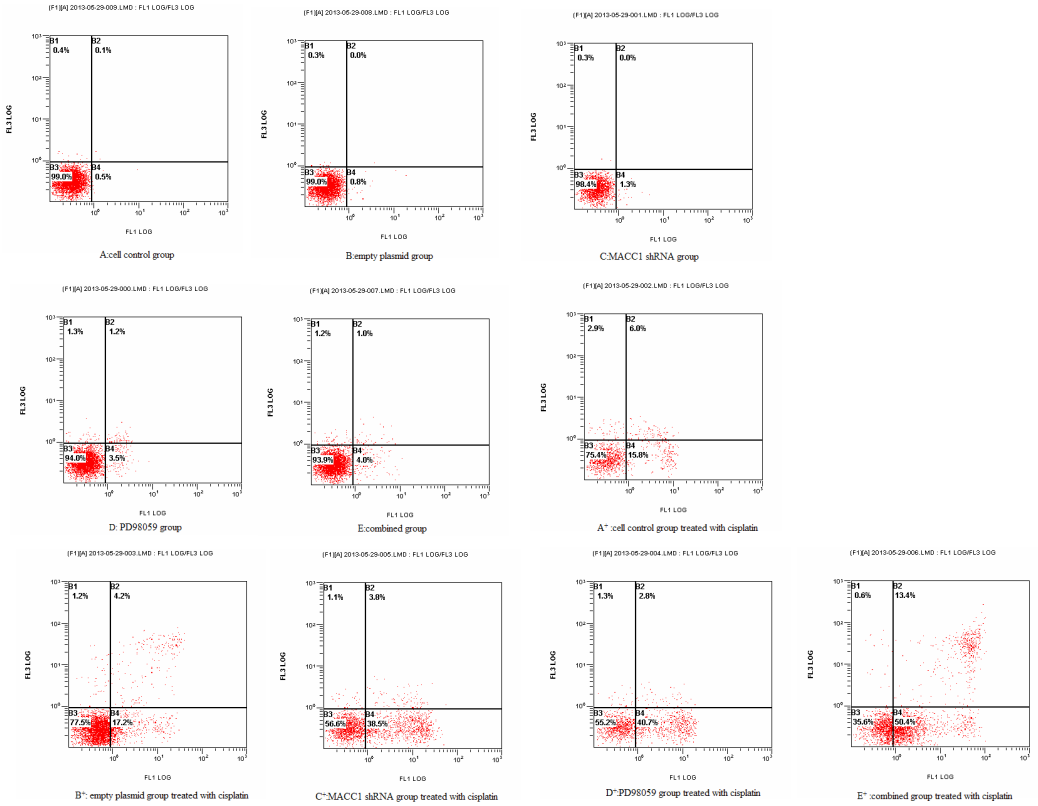

Figure 9. Cell apoptosis rate in 5 groups. **A.** Cell control group; **B.** empty plasmid group; **C.** pshMACC1 group; **D.** PD group; **E.** combined group; A+: cell control group treated with cisplatin; B+: empty plasmid group treated with cisplatin; C+: pshMACC1 group treated with cisplatin; D+: PD group treated with cisplatin; E+: combined group treated with cisplatin.

Table 1. Cisplatin-induced cell apoptosis in 5 groups.

	Control group (%)	shVect group (%)	pshMACC1 group (%)	PD group (%)	Combined group (%)
Without cisplatin treatment	0.48 ± 0.16	0.66 ± 0.36	1.32 ± 0.23	3.00 ± 0.26	3.70 ± 0.40
With cisplatin treatment	16.60 ± 1.20	18.53 ± 1.40	36.70 ± 0.95	38.17 ± 1.06	50.57 ± 0.45

Table 2. Effects of MACC1 on p-ERK1/2 expression in SKOV-3/DDP cells.

Groups	ERK1/2/β-actin	p-ERK1/2/β-actin
Control group	1.0381 ± 0.0052	0.9626 ± 0.0018
shVect group	1.0379 ± 0.0006	0.9625 ± 0.0056
pshMACC1 group	1.0381 ± 0.0002	0.8331 ± 0.0096*
PD group	1.0378 ± 0.0013	0.7431 ± 0.0000*
Combined group	1.0380 ± 0.0026	0.6776 ± 0.0041*

*P < 0.05, compared to the control group.

of drug resistance and improvement in drug sensitivity play a significant role in the treatment of ovarian cancer. The five-year survival rate of patients with ovarian cancer at the International Federation of Gynecology and Obstetrics stages (FIGO) I and II is about 70-90%. Most of the patients can be cured by surgery. However, the five-year survival rate of patients in FIGO stages III and IV is reduced to 20%.

MACC1 was first discovered by Stein in primary colon cancer and metastatic tissues (Stein et al., 2009). *MACC1*is located on chromosome 7p21.1 and consists of 2,559 nucleotides that encode a protein containing 852 amino acids (Stein et al., 2010). MACC1 protein contains a ZU5 domain, two hydroxyl terminal domains, and an SH3 domain. The tyrosine residues in the hydroxyl terminal domain are phosphorylated to trigger a cascade reaction and promote tumor invasion and metastasis when the DNA is damaged (Stein et al., 2009). *MACC1*, *HGF*, and C-*met* are believed to be over-expressed in ovarian epithelial cancer tissues; this expression pattern is associated with advanced FIGO stages, poor differentiation, and lymph node metastasis (Zhang et al., 2011b). MACC1 is also believed to functionas a potential biomarker and participate in the progression of ovarian cancer. Downregulation of MACC1 efficiently decreased C-met, p-ERK1/2, and p-MEK1/2 levels in ovarian carcinoma OVCAR-3cells (Zhang et al., 2011a). Furthermore, the inhibition of MACC1 by siRNA enhancesthe sensitivity ofpancreatic cancer cells to gemcitabine(Wang et al., 2012). MACC1 expression was downregulated by siRNAs to investigate the correlation between MACC1 and cisplatin resistance in SKOV-3/DDP cells. Our results showed that the downregulation of MACC1 in SKOV-3/DDP cells could inhibit cell proliferation and enhance cell apoptosis, which suggested that MACC1 might affect drug resistance and biological behavior of SKOV-3/DDP cells.

Hepatocyte growth factor (HGF) is a high-affinity ligand for the c-Met receptor tyrosine kinase. The HGF/c-MET signal transduction pathway plays an important role in cell proliferation, migration, angiogenesis, liver development, and regeneration. AberrantHGF/c-Met signaling may lead to tumor formation and metastasis (Comoglio et al., 2008). Mammals express multiple mitogen-activated protein kinase (MAPK) pathways, including the ERK1/2, JNK, P38, and ERK5/BMK1 pathways. ERK signaling plays an important role in the regulation of cell proliferation, survival, and apoptosis (Ramos, 2008). ERK is excessively activated in various types of malignant tumors and is considered to be an important target for the treatment of tumors (Roberts and Der, 2007). MACC1 might regulate tumor cell growth, apoptosis, invasion, metastasis, and drug resistance through the ERK signaling. Stein et al. (2009) reported that the over expression of MACC1 in colon cancer cells could activate ERK signaling and UO126 and PD98059 (ERK inhibitors) could inhibit HGF-mediated cell metastasis. *MACC1* gene silencing by siRNA also attenuated cell proliferation, invasion, and metastasis, and promoted cell apoptosis in OVCAR-3 cells. Moreover, there was a decrease in the level of phosphorylated ERK (Zhang et al., 2011a).

In this study, we attempted to determine whether*MACC1* regulated cisplatin sensitivity via ERK signaling in SKOV-3/DDP cells. The *MACC1* gene was silenced by transfecting SKOV-3/DDP cells with p-super-EGFP-shRNA plasmids. Our results showed that the downregulation of *MACC1* enhanced cisplatin sensitivity and decreased p-ERK1/2 levels in SKOV-3/DDP cells. Cisplatin sensitivity was enhanced in cells treated with 20 μM PD98059, suggesting that ERK inhibitors could improve cisplatin resistance in ovarian cancer cells. The results of thisstudy were consistent with those of previously reported studies (Wei et al., 2004). Moreover, the downregulation of MACC1 combined with PD98059 could further enhance cisplatin sensitivity.

Cisplatin exerts an anti-tumor effect by inducing DNA damage. Drug resistance is developed when the rate of cell apoptosis is decreased. Inhibition of cell apoptosis is one of the causes of

cisplatin resistance in ovarian cancer cells (Li et al., 2001). The results of our study suggested that MACC1 affected cisplatin sensitivity in ovarian cancer cells, probablythrough the ERK signal transduction pathway. Downregulation of MACC1 and blocking of the ERK signaling enhanced cisplatin sensitivity in a coordinated manner, which suggested a new method for improving cisplatin resistance in ovarian cancer. However, the regulation of cisplatin sensitivity by MACC1 via other signal transduction pathwaysremains to be studied.

Conflicts of interest

The authors declare no conflict of interest.

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