

Original Article

MCT1 promotes the cisplatin-resistance by antagonizing Fas in epithelial ovarian cancer

Chunxiao Yan¹, Fan Yang², Chunxia Zhou¹, Xuejun Chen¹, Xuechuan Han², Xueqin Liu², Hongyun Ma², Wei Zheng¹

¹Department of Gynecology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, People's Republic of China; ²Department of Obstetrics and Gynecology, Ningxia People's Hospital, Yinchuan 750002, People's Republic of China

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Abstract: This study was designed to investigate the role of MCT1 in the development of cisplatin-resistant ovarian cancer and its possible relationship with Fas. We found the expression of MCT1 was obviously increased both in cisplatin-resistant ovarian cancer tissue and A2780/CP cells compared with sensitive ovarian cancer tissue and cell lines A2780. And in A2780 cells treated with Cisplatin, the expression of MCT1 increased in a concentration-dependent manner, MCT1 knockdown attenuates cisplatin-induced cell viability. In A2780 and A2780/CP cells transfected with MCT1 siRNA, the activation of several downstream targets of Fas, including FasL and FAP-1 were largely prevented, whereas the expression of Caspase-3 was increased, accompanying with increased abundance of Fas. Coimmunoprecipitation and immunofluorescence showed that there is interaction between endogenous MCT1 with Fas in vivo and in vitro. In vivo, depletion of MCT1 by shRNA reverses cisplatin-resistance and the expression of Fas. This study showed that down regulation of MCT1 promote the sensibility to Cisplatin in ovarian cancer cell line. And this effect appeared to be mediated via antagonizing the effect of Fas.

Keywords: MCT1, Fas, cisplatin-resistant, epithelial ovarian cancer

Introduction

Epithelial ovarian cancer (EOC) is a major cause of morbidity and mortality among all gynecological malignancies [1]. As it is often asymptomatic in its early stages; approximately 75% of patients are diagnosed late in stage III or IV and < 30% of patients survive at 5 years. Treatment consists of aggressive primary debunking surgery followed by platinum-based chemotherapy in advanced disease. As we know, chemotherapeutic agents could induce apoptosis, and raising the intriguing possibility that cell death determinants may influence the outcome of treatment and chemoresistance [2, 3].

Drug resistance has been reported to be associated with several mechanisms, such as evasion of apoptosis, processing of drug-induced damage, drug inactivation or efflux and alteration in drug target [4-8]. And our previous study has proved that up-regulation of Fas could significantly promote cell apoptosis and migration

in cisplatin-resistant A2780/CP cells, and Fas might act as a promising therapeutic target for improvement of the sensibility to cisplatin in ovarian cancer [9]. But the exact mechanism is still unclear.

As we know, a variety of physiological functions in cells are inseparable from the energy supply. As the tumor cells growth faster and the metabolism also enhanced, the cells always lived in a hypoxic environment. The glycolysis plays as the main way of energy supply in tumor cells, so there are always a reduction of the Pasteur effect and an increasing of Crabtree effect [10]. And the function of lactic acid which is the main final metabolite of the glycolysis is extremely special. The accumulation of lactic acid in cells could affect the activity of the phosphofructokinase, which will in turn inhibit the glycolysis. Therefore, the transport body which is responsible for transferring lactic acid plays a key role in keeping cells' physiological function. MCT1 acted as a member of monocarboxylate transporters (MCTs) family which is a transmem-

brane transporter protein was found in ovarian cancer and many solid tumor cells, such as breast cancer and colorectal cancer [11, 12]. Except monocarboxylic acid, it also transports many other foreign substances, including hormone, medicine and something else. According to the important function of MCT1 which keep stable environment in cells, it is highly possible related to cisplatin-resistant in ovarian cancer.

Based on the research we have done before, we further investigated the role of MCT1 in the development of cisplatin-resistant ovarian cancer and its possible relationship with Fas.

Materials and methods

Reagents

MCT1, Fas, FasL, FAP-1, caspase-3, antibodies and agrose-protein A/G, IgG were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti rabbit-cy3, anti mouse-FITC were purchased from Jackson ImmunoResearch Company. DAB Horseradish Peroxidase Color Development Kit was purchased from Sigma-Aldrich (Sigma, USA). Cell Counting Assay Kit-8 (CCK-8) was purchased from Yiyuan Biotech (Yiyuanbiotech, China). Fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco (Gibco, USA). RNA interference reagent Hiper Fect and siRNA were purchased from Qiagen Sample & Assay Technologies (Qiagen, Germany).

Patients and samples

This study was approved by Ningxia people's hospital and all ethical guidelines were followed. An informed consent was obtained from every patient for participation in this study. In this prospective study, the case materials were obtained at first surgery from 30 patients (15 with chemo-resistance and 15 with sensitive ovarian cancer), who received histopathologic examination and diagnosed with malignant epithelial ovarian cancer. After surgery, the ovarian tissue was immersed in 10% buffered formalin for fixation at least 24 h. The paraffin-embedded blocks from the tissue were prepared. Serial section was cut on slides coated with poly-L-lysine (Sigma, USA) and stored for immunohistochemistry.

Immunohistochemistry

For the comparison of expression of Fas and MCT1, 15 chemo-resistance and 15 sensitive

ovarian cancer were included. Immunohistochemistry was done by the Biotin-Streptavidin-Peroxidase Complex method as described previously [13]. In brief, the sections prepared before were then deparaffinized in xylene and rehydrated to water through a graded series of alcohol (100%, 95% and 70%). The non-specific staining was blocked by incubating the sections with normal horse serum for 1 h. Then the primary antibodies against Fas (1:100, Santa Cruz) and MCT1 (1:100, Santa Cruz) were applied onto the sections which were then incubated overnight at 4°C. After that, incubated the sections with the biotinylated secondary antibody for 45 min at room temperature, washed three times with PBS and incubated in streptavidin-peroxidase complex reagent for another 45 min and then in peroxidase substrate solution, i.e., with 3-3' diaminobenzidine tetrahydrochloride (DAB) (Sigma, USA) for 5-10 min. The endogenous peroxidase activity was blocked by incubating the sections with the blocking solution (3% H₂O₂ in methanol) for 20 min. The sections were then rinsed in tap water, counter stained with hematoxylin, and mounted with DPX. The brown product obtained was visualized and scored by light microscopy. The omission of the primary antibody was used as the negative control.

The immunohistochemistry (IHC) score was recorded by multiplying the percentage positivity with intensity score obtained. IHC score = % age positivity × intensity score.

Cell culture

The human epithelial ovarian cancer cell line A2780 were cultured in RPMI 1640 medium containing 10% new born serum (FBS, Gibco, USA), 50 U/ml penicillin and 50 U/ml streptomycin (Gibco, USA) in a humidified atmosphere of 5% CO₂ at 37°C. And the Cisplatin-resistant A2780 (A2780/CP) cells were generated by exposure to increasing concentration of Cisplatin according to literature.

Cell counting kit-8 (CCK-8) assay for cisplatin sensitivity

Cisplatin sensitivity was measured by cell counting assay kit-8 (Yiyuanbiotech, Guangzhou, China) according to the manufacturer's protocol. 100 µL of cells were plated on 96-well plates at a density of 1~1.5 × 10⁴ cells/ml for

24 h followed by a cell synchrotronization, and treated with cisplatin (0, 10, 20 μ M) for 48 h. Then 10 μ L of CCK-8 solution was added to each well for another 2 h, and the absorbance at 450 nm was measured by a microplate reader (Bio-Tek, Winooski, VT, USA).

Immunofluorescence analysis

Immunofluorescence (IF) analysis of MCT1 and Fas was performed as described previously [15]. In brief, cells were plated in confocal dishes for 24 h. Then fixed with 4% paraformaldehyde solution for 10 min, before and after washed three times with PBS, And permeabilized with 0.01% Triton X-100 for 1 min. Following permeabilization, cells were incubated with 5% BSA for 30 min at room temperature. Cells were then incubated with the primary antibodies either against Fas (1:100, Santa Cruz) or MCT1 (1:100, Santa Cruz) overnight at 4°C. After that, incubate the cells with the appropriate fluorescent secondary antibody for 45 min at room temperature followed by a wash with PBS darkly. Cells were viewed using a confocal laser scanning microscope (Olympus FV500 + IX81, JP).

Coimmunoprecipitation

The potential interaction between MCT1 and Fas were confirmed by coimmunoprecipitation in cisplatin-resistant A2780/CP cells. Lyse Cells after washed twice with ice-cold PBS. Cells were gently scraped with a rubber policeman before being centrifuged at 12000 \times g/min for 10 min at 4°C. Protein concentrations were determined by Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), with bovin serum albumin (BSA) as the standard. Then Samples containing 200 μ g of protein were incubated with primary antibodies against Fas (1:100, Santa Cruz) overnight at 4°C. The IgG antibody was used as the negative control. Then added 20 μ L agarose-protein A/G beads in each sample gently rocking for 4 h at 4°C and followed with centrifuge. Remove the screw cap, place the column into a new tube, add 200 μ L of IP Lysis/Wash Buffer and centrifuge. Wash the sample two more times with 500 μ L IP Lysis and centrifuge after each wash. Equilibrate the sample with loading buffer and heated at 95-100°C for ~5 minutes. Western blot was used for further analysis.

Transfection of A2780/CP and A2780 with siRNA

The sequence of the stealth siRNA duplex oligoribonucleotides against human MCT gene is obtained from GenBank. The MCT siRNA was transfected with Lipofectamine 2000 reagent according to the manufacturer (Invitrogen, Life Technologies, Inc.). And a negative stealth siRNA sequence was used as the control. The expression of MCT protein was detected by Western blot analysis.

Western blot analysis

Western blot analysis was performed as described previously [14]. Briefly, cultured cells were washed twice with ice-cold PBS and lysed in 80 μ L lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.02% NaN₃, 1% nonidet P-40, 0.1% sodium dodecyl sulfate, sodium deoxycholate, and 1% protease inhibitor cocktail), and then incubated on ice for approximately 30 minutes. Cells were gently scraped with a rubber policeman before being centrifuged at 12000 \times g/min for 12 min at 4°C. Protein concentrations were determined by Bradford method (Bio-Rad Laboratories, Hercules, CA, USA), with bovin serum albumin (BSA) as the standard.

Samples containing 60 μ g of protein were subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked at room temperature for 1h in 5% non-fat dry milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20), and incubated with primary antibodies overnight at 4°C and then with the appropriate secondary peroxidase-conjugated antibodies (HRP-linked anti-rabbit secondary antibody) for 1.5 h at room temperature. Blot were developed using a chemiluminescence system (Cell Signaling Technology Inc., Beverly, Mass.). They were then visualized by exposure to Kodak X-ray film. The result were scanned and analyzed with Image J software.

Xenograft formation and in vivo research

MCT1 shRNA constructs in adenovirus vector was purchased from Invitrogen (Life Technologies, Inc.). And the sequences of the MCT1 shRNA were as follows: Top, 5'-CACCGCAGTATCCTGTGAATAAATCGAAATTTATTCACCAGGATACTGC-3'; Bottom, 5'-AAAAGCAGTATCCTGGTGAATAAATTCGATTATTCACCAGGATACTGC-3'.

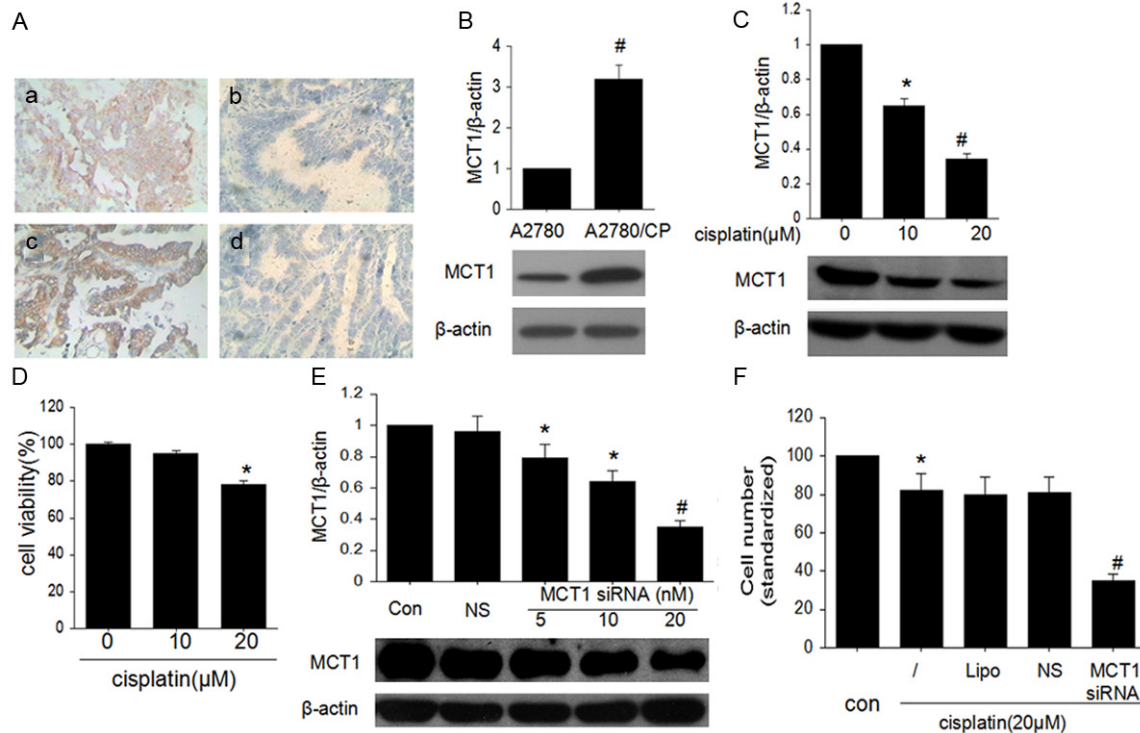


Figure 1. The expression of MCT1 is correlated with cisplatin-resistance in epithelial ovarian tumors and MCT1 expression parallels with rate of A2780 cells proliferation. (A) Immunohistochemical protein expression of MCT1 and Fas in chemo-resistance (Aa, C) versus sensitive (B, D) epithelial ovarian tumors. (a, b) MCT1; (c, d) Fas (OM. $\times 400$); (B) MCT1 expression in cisplatin-resistant A2780/CP cells and parent A2780 cells detected by western blot. (The data shown are mean \pm SEM (n = 3). # $P < 0.01$ vs. A2780 group); (C) Expression of MCT1 in A2780 treated with Cisplatin in different concentration (0, 10, 20 μ M). (The data shown are mean \pm SEM (n = 3). 0 represents vehicle DMSO; * $P < 0.05$ vs. 0 group, # $P < 0.01$ vs. 0 group); (D) cell viability in A2780 after treated with cisplatin (0, 10, 20 μ M) for 48 h. (The data shown are mean \pm SEM (n = 3). 0 represents vehicle DMSO; * $P < 0.05$ vs. 0 group); (E) Screen of MCT1 siRNA concentration in A2780 cells. (The data shown are mean \pm SEM (n = 3). con represents control; NS represents negative control; * $P < 0.05$ vs. con group, # $P < 0.05$ vs. con group); (F) Effect of MCT1 siRNA on cisplatin-induced cell viability in A2780 cells. The data shown are mean \pm SEM (n = 6). con represents control, Lipo represents empty lipoplast, NS represents negative control; * $P < 0.05$ vs. con group; # $P < 0.01$ vs. cisplatin only).

We used 4 weeks old female athymic BALB/c nude mice (Vital River Laboratories, Beijing, China). 2.5×10^7 of A2780/CP cells that suspended in 1ml antibiotic-free PBS were injected subcutaneously into the left side neck of the mice. After one week, we can see an obvious tumor at the injection site. When the size of the tumors reached 50 mm³, mice were randomly divided into four groups with six mice in each group: three treatment group (cisplatin 4 mg/kg combined with 100 MOI MCT1 shRNA were injected via tail vein route per 3 days for 30 days); the ad-laz control group (cisplatin 4 mg/kg combined with an equal volume of ad-laz was injected the same route at the same time); the control group (only cisplatin 4 mg/kg was injected the same route at the same time); and the normal group (an equal volume of PBS was injected the same route at the same time).

Every time before injection we measured the tumor dimensions with digital vernier caliper (the tumor size = $\pi/6 \times \text{length} \times (\text{width})^2$). All the animal experiments were approved by Ningxia Medical University Committee for Animal Research and performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of China, Beijing).

Statistical analysis

All of the values were expressed as the mean \pm SEM. Comparisons between 2 groups were analyzed using Student's t test and among 3 groups by ANOVA followed by a post-hoc comparison using the least significant difference test (SPSS 13.0). Values of $P < 0.05$ were considered statistically significant.

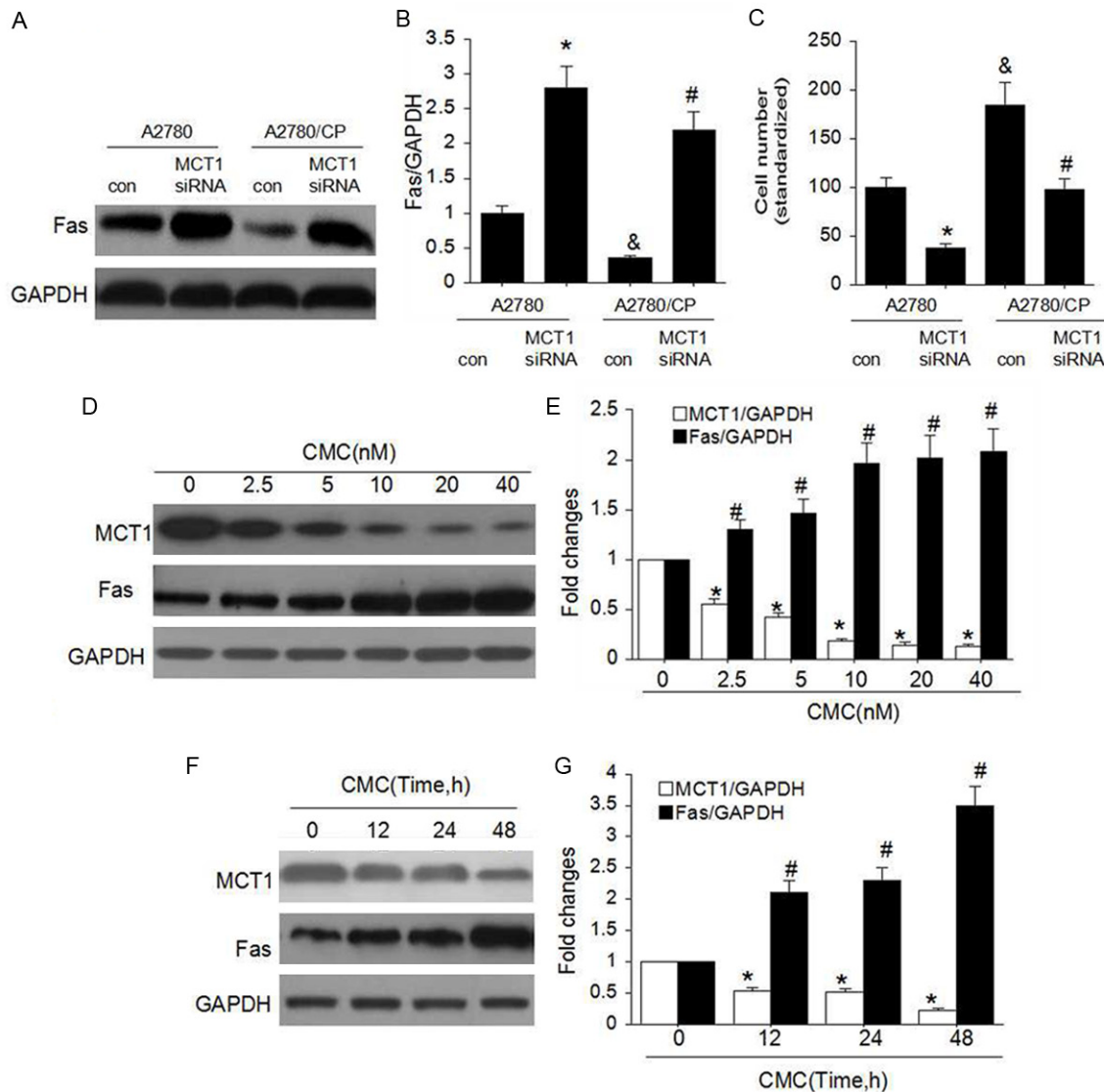


Figure 2. MCT1 downregulation enhance expression of Fas. A and B. Expression of Fas in A2780 and A2780/CP cells transiently transfected with MCT1siRNA. (The data shown are mean \pm SEM (n = 6). *P < 0.05 vs. A2780 con group, #P < 0.01 vs. A2780 con group, &P < 0.05 vs. A2780 con group); C. Effect of MCT1 siRNA on cell viability in A2780 and A2780/CP cells. (The data shown are mean \pm SEM (n = 6). *P < 0.05 vs. A2780 con group, #P < 0.01 vs. A2780 con group, &P < 0.05 vs. A2780 con group); D and E. Effect of 0-20 nM CHC treatment for 48 h on the expression of MCT1 and Fas. (The data shown are mean \pm SEM (n = 6). 0 represents vehicle DMSO; *P < 0.05 vs. 0 group, #P < 0.01 vs. 0 group); F and G. Effect of 10 nM CHC on the expression of MCT1 and Fas at 0-48 h. (The data shown are mean \pm SEM (n = 6). 0 represents vehicle DMSO; *P < 0.05 vs. 0 group, #P < 0.01 vs. 0 group).

Results

Expression of MCT1 is correlated with cisplatin-resistance in epithelial ovarian tumors

Immunohistochemistry was performed to evaluate the protein expression in ovarian cancer tissues, and western blot was used to detect the protein expression on cellular level. As shown in **Figure 1A**, the expression of MCT1 was higher in chemo-resistance ovarian tis-

sues, accompanying with a lower expression of Fas. And MCT1 expression (**Figure 1B**) was remarkably increased in cisplatin-resistant A2780/CP cells with comparing to parent A2780 cells. After treatment with various concentration of cisplatin (0, 10, 20 μ M), the expression of MCT1 increased in a concentration-dependent manner in A2780 cells (**Figure 1C, 1D**). The small interfere RNA targeting at MCT1 could obviously decreased endogenous MCT1 expression in A2780 cells (**Figure 1E**).

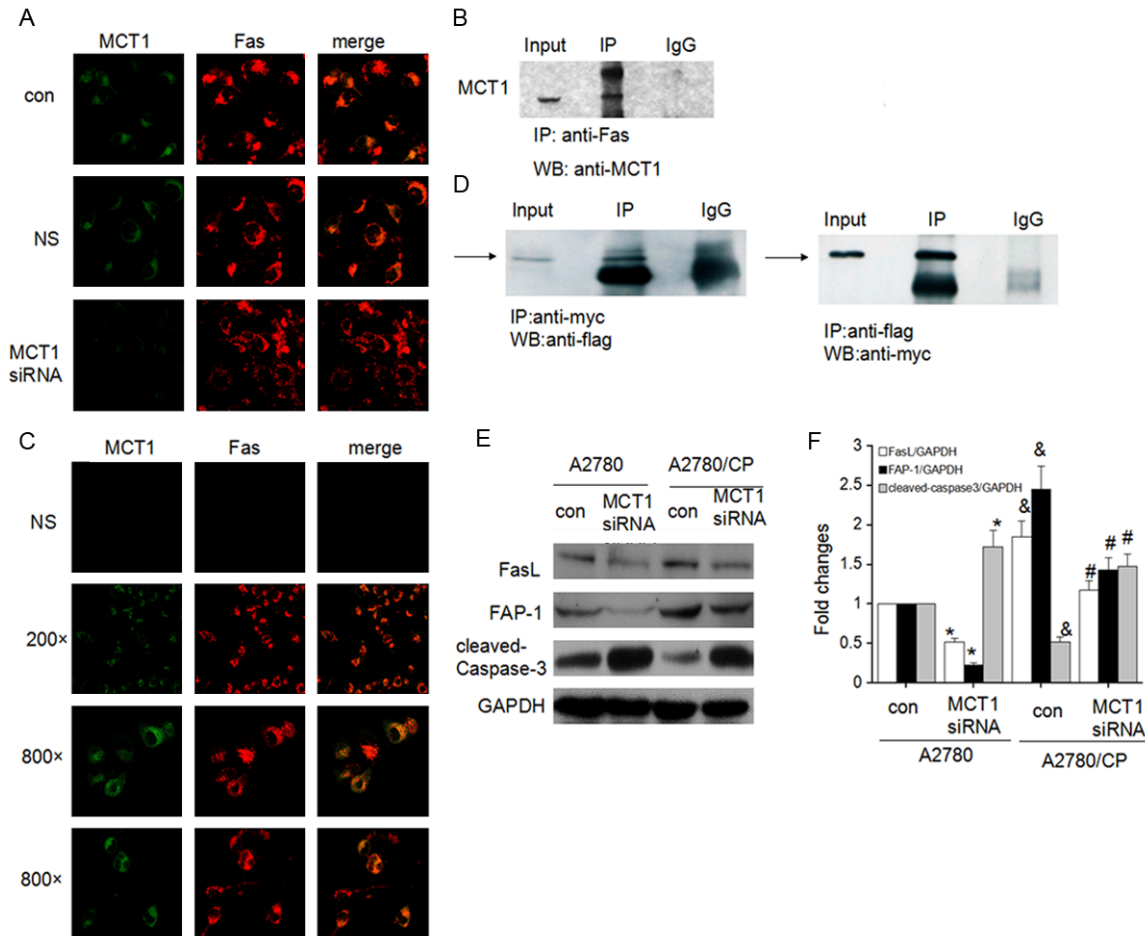


Figure 3. Interaction of MCT1 with Fas. A. Effect of MCT1 siRNA knockdown on the expression of MCT1 and Fas by immunofluorescence (800 ×); B. Coimmunoprecipitation of MCT1 with Fas from A2780/CP cell lysates (n = 6). Cell lysate was immunoprecipitated (IP) by anti-Fas antibody (IP) or not (input), and analyzed by western blot (WB) with anti-MCT1 antibody; C. The co-localization between MCT1 and Fas detected by dual immunofluorescent staining; D. Direct binding between MCT1 and Fas in vitro (n = 6); E and F. Effect of MCT1 knockdown on the activity of Fas/FasL pathway. After transfected with MCT1 siRNA for 48 h, cells were harvested and lysed, then the indicate proteins were detected by Western blot. (The data shown are mean ± SEM (n = 6). **P* < 0.05 vs. A2780 con group, &*P* < 0.01 vs. A2780 con group, &*P* < 0.05 vs. A2780 con group).

And MCT1 knockdown attenuates cisplatin-induced cell viability (**Figure 1F**). These results indicated that increasing expression of MCT1 might be associated with cisplatin-resistance in ovarian cancer.

MCT1 downregulation enhance expression of Fas

In **Figure 2A, 2B**, Western Blot results showed that Fas protein expression was significant increased in MCT1 siRNA group both in A2780 and A2780/CP cells. MCT1 knockdown attenuates cisplatin-induced cell viability both in A2780 and A2780/CP cells, but this effect was enlarged in A2780/CP cells (**Figure 2C**).

Moreover, we used a highly selective inhibitor of MCT1 to study the function of MCT1 [15]. As expected, we observed a dose-dependent (**Figure 2D, 2E**) and time-dependent (**Figure 2F, 2G**) down-regulation of MCT1 after CHC treatment in A2780 cells, accompanying with enhanced expression of Fas. These results prompt that MCT1 and Fas may have interaction in cells.

Interaction between MCT1 and Fas

Coimmunoprecipitation and immunofluorescence showed that there is interaction between endogenous MCT1 with Fas in A2780/CP cells (**Figure 3A, 3B**). Furthermore, to confirm the

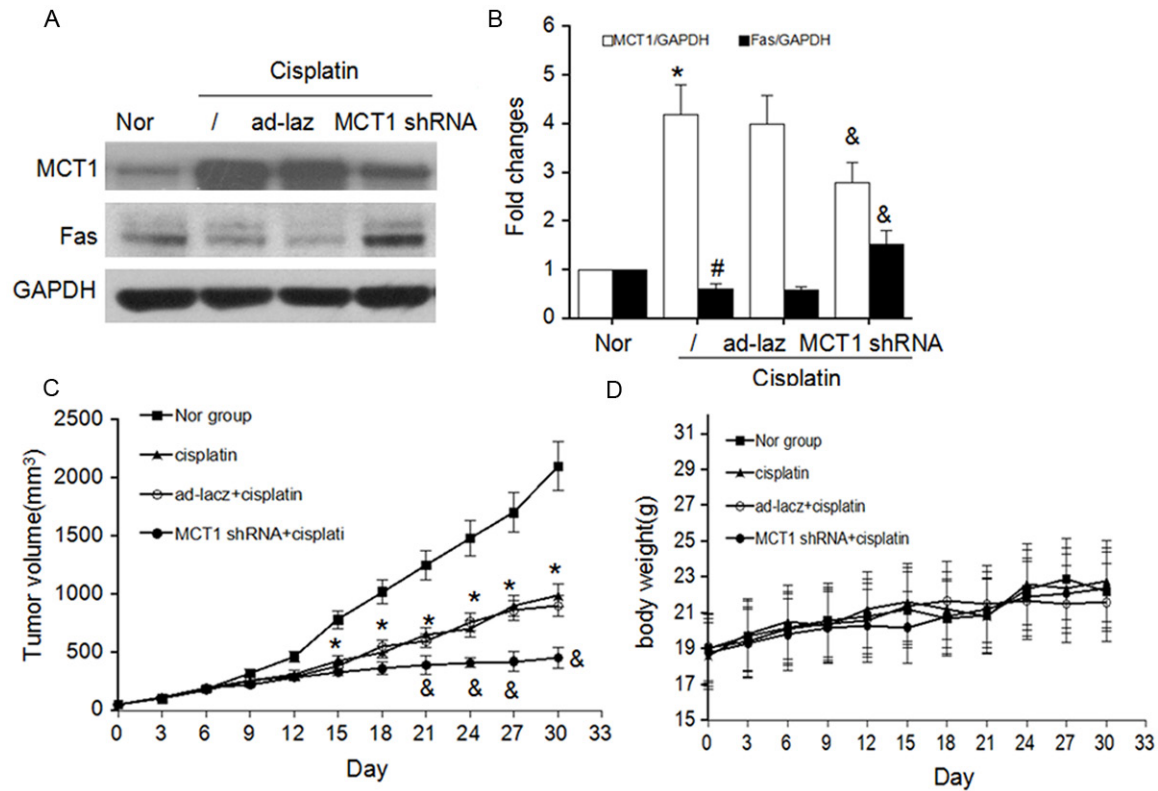


Figure 4. Depletion of MCT1 reverses cisplatin-resistance in epithelial ovarian tumor in vivo. A and B. depletion of MCT1 increases the expression of Fas in vivo. (The data shown are mean \pm SEM (n = 6). Nor represents Normal; * $P < 0.05$ vs. Nor group, & $P < 0.05$ vs. cisplatin only group); C. representative images of tumors on day 30 treated with cisplatin alone or combined with 100 MOI MCT1 shRNA vector group (The data shown are mean \pm SEM (n = 6). Nor represents Normal; * $P < 0.05$ vs. Nor group, & $P < 0.05$ vs. cisplatin only group); D. changes of mice body weight during the dosage.

binding of MCT1 and Fas in vitro, we constructed MCT1-flag and Fas-myc plasmid. And cotransfection MCT1-flag with Fas-myc plasmid respectively, coimmunoprecipitation and immunofluorescence results proved that exogenous MCT1 and Fas interacted with each other in an in vitro binding assay (Figure 3C, 3D).

Effects of MCT1 in Fas/FasL pathway

To further investigate the relationship between MCT1 and Fas, we detected the activation of Fas and the expression several downstream targets of Fas, including FasL, FAP-1, and Caspase-3. Western Blot results (Figure 3E, 3F) showed that In A2780/CP cells transfected with MCT1 siRNA, the activation of several downstream targets of Fas, including FasL and FAP-1 were largely prevented ($P < 0.05$) and the expression of Caspase-3 was increased, accompanying with decreased abundance of MCT1. All these results suggest that MCT1 could antagonize the activation of Fas/FasL pathway.

Depletion of MCT1 reverses cisplatin-resistance in epithelial ovarian tumor in vivo

To evaluate whether MCT1 knockdown could inhibit cisplatin-resistance of epithelial ovarian cancer in vivo, we established the xenograft model of human epithelial ovarian cancer in nude mice, and then injected cisplatin alone or combined with 25, 50, 100 MOI MCT1 shRNA. As it is showed in Figure 4A, 4B, the expression of MCT1 was significantly decreased in the treated group, suggesting that the recombinant adenovirus vector Ad-SH3GL1 shRNA was effective in vivo. And in line with our research in vitro, the expression of Fas was up-regulation with the depletion of MCT1. Then we found that injection with 100 MOI shRNA could suppressed the tumor growth remarkably than those treated with cisplatin alone (Figure 4C). During the course of treatment, no significant changes were observed of mice body weight among all groups (Figure 4D), which suggests

that at the treatment dosage there was no significant toxicity to mice.

Discussion

Increased use of the glycolytic pathway is considered to resulting in a selective advantage for tumor cells; however, it often accompanying with increased production of lactic acid, which may break the steady intracellular environment and result in cell death [16]. Consequently, upregulation of MCT family proteins which is the main protein linked with proton export, such as MCT1 and MCT4, has been demonstrated in numerous solid tumors [18-20]. But there is little report about the role of MCT family proteins in the development of drug-resistant. In the present study, we explored the role of MCT1 in the development of cisplatin-resistant ovarian cancer and its possible relationship with Fas.

Our results showed that the expression of MCT1 was obviously increased both in cisplatin-resistant ovarian cancer tissue and A2780/CP cells compared with sensitive ovarian cancer tissue and cell lines A2780. Interestingly, in A2780 cells treated with Cisplatin, the expression of MCT1 increased in a concentration-dependent manner and MCT1 knockout attenuates cisplatin-induced cell viability. These results indicated that increasing expression of MCT1 is associated with cisplatin-resistance in ovarian cancer.

Cell abnormal proliferation and apoptosis contributed to pathogenesis of cancer. And according to this point, cisplatin-resistance might also relate to the imbalance between cell proliferation and apoptosis. As we know, there are two major signaling pathways mediate cell apoptosis: the death receptor-mediated pathway and the mitochondrial pathway [16]. Accumulating of lactic acid may due to cell death [17, 21, 22], and our previous study showed that Fas might act as a promising therapeutic target for improvement of the sensibility to Cisplatin in ovarian cancer [9]. We assumed that MCT1 may be affect cell death pathway, and our present study consistence with this hypothesis, we found that there is an interaction between MCT1 and Fas both in vivo and in vitro. We further examined whether Fas/FasL pathway was involved in the cisplatin-resistance of ovarian cancer regulated by MCT1. In A2780/CP cells

transfected with MCT1 siRNA, the activation of several downstream targets of Fas, including FasL and FAP-1 were largely prevented and the expression of Caspase-3 was increased, accompanying with decreased abundance of MCT1. All these results suggest that MCT1 could antagonize the activation of Fas/FasL pathway. And these may means MCT1 could antagonize the activation of Fas/FasL pathway and promote Cisplatin-resistance the in ovarian cancer cell line. In vivo, depletion of MCT1 by shRNA reverses cisplatin-resistance and the expression of Fas. In conclusion, our current study demonstrated that down regulation of MCT1 promote the sensibility to Cisplatin in ovarian cancer cell line. And this effect appeared to be mediated via antagonizing the effect of Fas.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Wei Zheng, Department of Gynecology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, 88 Jiefang Road, Hangzhou 310009, Zhejiang Province, China, Tel:+86-571-87783738; Fax: +86-571-87783738; E-mail: drzhengwei@126.com

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