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The *ABCB1* 3435C > T polymorphism influences docetaxel transportation in ovarian cancer

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Abstract

Objective: To investigate the effect of the ATP-binding cassette transporter superfamily B member 1 gene (*ABCB1*) 3435C > T single nucleotide polymorphism (SNP) on docetaxel transportation in ovarian cancer cells.

Methods: ES-2 and SKOV3 cells were transfected with an *ABCB1* 3435C > T recombinant plasmid, and mRNA expression was detected by real-time PCR. The MTT assay was used to detect the toxicity of docetaxel. High-performance liquid chromatography determined the drug concentration in different cell models to evaluate intracellular accumulation, and a transmembrane resistance experiment was used to assess permeability and evaluate the effect of P-gp activity on drug transportation. A tumor-bearing mouse model was established to evaluate the effect of *ABCB1* 3435C > T on docetaxel resistance.

Results: P-gp was overexpressed in cells transfected with the *ABCB1* 3435C > T plasmid, leading to a significant increase in drug resistance to docetaxel. *ABCB1* 3435C/wild-type transfection significantly promoted the transport of docetaxel mediated by P-gp compared with *ABCB1* 3435T/mutant transfection.

Conclusion: P-gp encoded by the *ABCB1* variant allele appears to be more efficient at transporting docetaxel compared with the wild-type allele. The *ABCB1* 3435C > T SNP dramatically affected the efflux ability of P-gp against docetaxel, and may influence P-gp expression and activity.

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Introduction

Docetaxel is a major anti-tumor drug used for the treatment of malignant tumors such as breast cancer, ovarian cancer, and non-small cell lung cancer. The remarkable efficacy of the drug makes it widely applicable, and its metabolism and clearance have received much attention.[1,2] However, there are significant differences in the clinical pharmacokinetics and pharmacodynamics among individuals, and efficacy and adverse reactions also vary.

P-glycoprotein (P-gp) is a member of the ABC transporter family. Its main function is to pump substrates such as docetaxel out of the cell, and it is also involved in substrate absorption and distribution. P-gp is an important transporter *in vivo* that is widely distributed in the liver, brain, kidney, gastrointestinal tract, and other tissues. P-gp overexpression is the main cause of multidrug resistance in various human tissues.[3] It is encoded by the ATP-binding cassette transporter superfamily B member 1 gene (*ABCB1*), which is over 100 kb, is localized on chromosome 7q21.1, and contains 28 exons. *ABCB1* is highly expressed in various tumor tissues.[4–6] Currently, more than 60 single nucleotide polymorphism (SNPs) in *ABCB1* have been reported, with the most common being 1236 C > T, 2677G > T/A, and 3435C > T.

Different *ABCB1*SNPs can significantly influence the efficacy and adverse effects of paclitaxel.[7,8] Vaclavikova et al.[9] found that *ABCB1* expression was down-regulated in 79.5% of cancer tissues, and was associated with the polymorphic mutations C3435T and C1236T. Lal et al.[10] found that the clearance rate of paclitaxel in CC-GG-CC wild-type patients with C1236T, G2677T/A, and C3435T mutations was significantly increased compared with CT-GT-CT and TT-TT-TT mutants, while the plasma peak drug concentration was significantly decreased. Conversely, Fransson et al.[11] reported that SNPs have only a small influence on the plasma concentration of paclitaxel, but that even a slight change of transfer or metabolism would affect the concentration of the main metabolic products of paclitaxel. The effect of *ABCB1* on drug toxicity is therefore of greater importance than the pharmacokinetics of the drug itself. A study in Caucasian female patients with ovarian cancer[12] found that the clearance rates of paclitaxel had no significant correlation with *ABCB1* C1236T or its variants. However, only limited reports are available on the effect of SNPs on drug sensitivity in ovarian cancer cells.

In this study, *ABCB1* (3435C > T) recombinant plasmids were constructed and transfected into docetaxel-resistant ES-2 and SKOV3 cells. The effect of *ABCB1* (3435C > T) polymorphisms on the expression level of P-gp and the correlation of SNPs with drug susceptibility were investigated. Our findings provide a theoretical guidance for ovarian cancer clinical practice.

Materials and methods

Cell culture

ES-2 and SKOV3 cells, purchased from the Cell Bank of Type Culture Collection of the

Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's modified Eagle medium containing 1 g/L glucose and glutamine (GIBCO® Cell Culture, Carlsbad, CA, USA), 10% fetal bovine serum (GIBCO), and 1% neomycin (Gibco), at 37°C in an atmosphere of 5% CO_2. Cells were seeded in a six-well plate at a density of 2000 cells/well and were assessed after 24 to 120 hours.

Construction of recombinant plasmids and cell transfection

The QuikChange II XL Site-directed Mutagenesis Kit (Agilent Technologies Inc., Santa Clara, CA, USA) was used to perform site-directed mutagenesis in the eukaryotic expression vector pcDNA 3.1 (GeneChem, Shanghai, China) to construct two recombinant plasmids: wild-type plasmid *ABCB1* 3435 C/wt and the mutant plasmid *ABCB1* 3435 T/mut. The point mutation primer was designed by Sangon Biotech (Shanghai, China). Sequencing and validation of recombinant plasmids were performed by Sanger sequencing (GATC Biotech AG, Konstanz, Germany).

ES-2 and SKOV3 cells ($6–8 \times 10^6$ cells) were seeded in 25-cm culture dishes. The two recombinant plasmids (10 μg each) were transfected into both cell lines using Lipofectamine 2000 (Thermo Fisher Scientific Inc., Rockford, IL, USA). At 48 hours after transformation, 8 μg/mL puromycin was added to screen for stable transformants.

Western blot

Radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitors was added to cells for protein extraction. The protein concentration was measured using a BCA kit. Samples were denatured and electrophoresed using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Subsequently, proteins were transferred to polyvinylidene fluoride membranes and probed with rabbit anti-P-gp (1:1000; Sigma-Aldrich, St. Louis, MO, USA) and mouse anti-GAPDH (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibodies overnight at 4°C. Membranes were then incubated with anti-mouse (1:5000, Santa Cruz Biotechnology) and anti-rabbit secondary antibodies (1:5000, Santa Cruz Biotechnology), respectively, for 1 hour. Finally, immunoreactive bands were detected with Enhanced Chemiluminescence Plus reagent (Bio-Rad, Hercules, CA, USA). Band densities were analyzed with Quantity One software (Bio-Rad) and used to determine protein expression relative to that of GAPDH.

Real-time (RT-)PCR

To determine changes in P-gp mRNA expression, cDNA synthesis was performed using an RNA PCR kit (Takara Bio Inc., Shiga, Japan). Quantitative real-time PCR was carried out using a SYBR premix Ex TaqII kit (Takara Bio Inc.) according to the manufacturer's instructions. Beta-actin was used as an internal control. RT-PCR was performed using a 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the threshold cycle (CT) number was determined using ABI 7500 Real-time PCR System SDS software v2.0.1. All reactions were performed in triplicate. Relative quantification of P-gp expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer information is given in Table 1.

MTT assay

The MTT assay was used to detect cell proliferation. Briefly, cells were seeded at a density of 1×10^4 cells/well of a 96-well plate and cultured for 48 hours. Each well was supplemented with 7.0 μmol/L

Table 1. Information about primers used in the study.

Gene name and orientation	Primer sequence (5′–3′)	Primer length (bp)	Amplicon length (bp)
MDR1			
Sense	GGCACCAAAGACAACAGCTG	20	286
Anti-sense	ATGTCCTTTTCCAGCACCTC	20	
β-actin			
Sense	TGTTTGAGACCTTCAACACCC	21	529
Anti-sense	AGCACTGTGTTGGCGTACAG	20	

docetaxel (Qilu Pharmaceutical Co. Ltd., Jinan, China), and 15 replicates were included for each group. Every 24 hours, cells from three replicates of each group were assessed by the MTT assay. The absorbance was read at 490/570 nm using a microplate reader. After obtaining optical density values for 5 days, the growth curve of cells under docetaxel selection was drawn.

Drug accumulation test

Cells were seeded in six-well plates at 5.0×10^5 cells/well and cultured for 4 hours. When cells were in the logarithmic phase, serum-free Dulbecco's modified Eagle's medium and 6.0 μmol/L docetaxel were added. Then, cyclosporin A (0.5 and 1.0 μM; Novartis Pharma AG, Basel, Switzerland) was added to inhibit drug transportation. After incubation at 37°C for 4 hours, cells were collected, washed with phosphate-buffered saline, centrifuged at $800 \times g$ for 5 minutes, and the supernatant was discarded. The cell pellet was stored at −80°C until required.

Quantitative analysis of cell drug accumulation was performed using high-performance liquid chromatography (HPLC) as previously described.[13] Briefly, 150 μL RIPA lysis buffer was added to cells and incubated in an ice bath for 30 minutes. Docetaxel was then extracted with glacial acetic acid and ethyl acetate. The supernatant was collected, and the solvents were evaporated using a nitrogen-drying apparatus (Ember, Shanghai, China). The obtained residue was dissolved in ethanol, then injected into the Agilent 1100 HPLC system (Waters Corp., Milford, MA, USA) with Agilent 4.6 mm × 250 mm columns (Waters Corp.). The amount of drug extracted from the cells was calculated using a standard curve (1.0–50 μM) and normalized to the total protein content of the cells.

Transmembrane transport analysis

The bidirectional transfer test was performed using cells seeded at a density of 1×10^4/well in a Transwell™ plate (3 μm). The transepithelial electrical resistance was measured using a Millicell electrical resistance system (Millipore, Billerica, MA, USA). To evaluate anti-tumor drug transport mediated by P-gp (A→B), HBSS-GH solution containing 7.0 μM docetaxel was added to the upper chamber (A) of the Transwell apparatus, and HBSS-GH was added to the lower chamber (B). Transport from B to A was used to measure the drug concentration from B to A. HBSS-FH solution containing 1.0 μmol/L cyclosporine A was added to upper and lower chambers to inhibit the activity of P-gp. According to previous studies,[6,14–18] the apparent permeability value (Papp) was calculated by $\text{Papp} = (\Delta Q/\Delta t)/(C_0 \times A)$, where $\Delta Q/\Delta t$ is the rate of drug transported to the receiving chamber; C_0 is the initial

concentration of the drug; and A is the permeability surface area. Both Papp of B→A and that of A→B were calculated. Each test was performed in triplicate.

In vivo assay

Male athymic nude mice (BALB/c-nu/nu) aged 4 to 6 weeks and weighing 18 to 22 g were purchased from Shanghai Slike Experimental Animals Co. (Shanghai, China). The mice were cared for according to Animal Facility Guidelines of the China Pharmaceutical University. Mice were randomized into three groups (n=10 per group): M0, control group; M1, implanted with SKOV3 cells transfected with *ABCB1* 3435 C/wt plasmids; and M2, implanted with SKOV3 cells transfected with *ABCB1* 3435T/mut plasmids. Cells were implanted into the subcutaneous tissue of the right groin of the mice. When the tumor volumes exceeded 100 mm^3, docetaxel (8 mg/kg) was administered every 4 days for a total of five times. The animal body weight was recorded daily. On the last day (Day 21), mice were sacrificed after treatment, and their plasma and tissues were collected and stored at −80°C until analysis. The study protocol was approved by the Ethics Committee of Shandong Provincial Qianfoshan Hospital, Shandong University.

Statistical analysis

All experiments were performed in triplicate. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data. Measurement data were expressed as means ± standard deviations (SDs). Comparisons between groups were analyzed by the Student's *t*-test. A P value less than 0.05 was considered statistically significant.

Results

Effect of the ABCB1 (3435C > T) polymorphism on P-gp protein expression

To investigate P-gp expression in cells, western blotting was performed. P-gp expression in ES-2 cells was increased 3.8-fold compared with the control group while that in SKOV3 cells was increased 7-fold (Figure 1).

Effect of the ABCB1 (3435C > T) polymorphism on P-gp mRNA expression

RT-PCR was performed to detect *P-gp* mRNA expression in cells. The expression of *P-gp* mRNA in both ES-2 and SKOV3 cells stably transfected with *ABCB1* 3435 C/wt and *ABCB1* 3435T/mut was significantly higher than in control cells ($P < 0.01$, Figure 2). *P-gp* mRNA was overexpressed in transfected cell lines, which was consistent with western blot findings.

Effect of the ABCB1 (3435C > T) polymorphism on docetaxel cytotoxicity

The MTT assay was performed to determine the effect of docetaxel on cell proliferation. In both ES-2 and SKOV3 cell lines, the survival of cells stably transfected with *ABCB1* 3435T/mut was increased 1.11-fold compared with cells stably transfected with *ABCB1* 3435 C/wt (Figure 3). Additionally, compared with the control group, cells stably transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut showed significantly higher cell survival ($P < 0.05$). This suggests that the drug resistance of cells to docetaxel is increased after *ABCB1* (3435C > T) transfection and that *ABCB1* SNPs are closely associated with cell sensitivity to drugs.

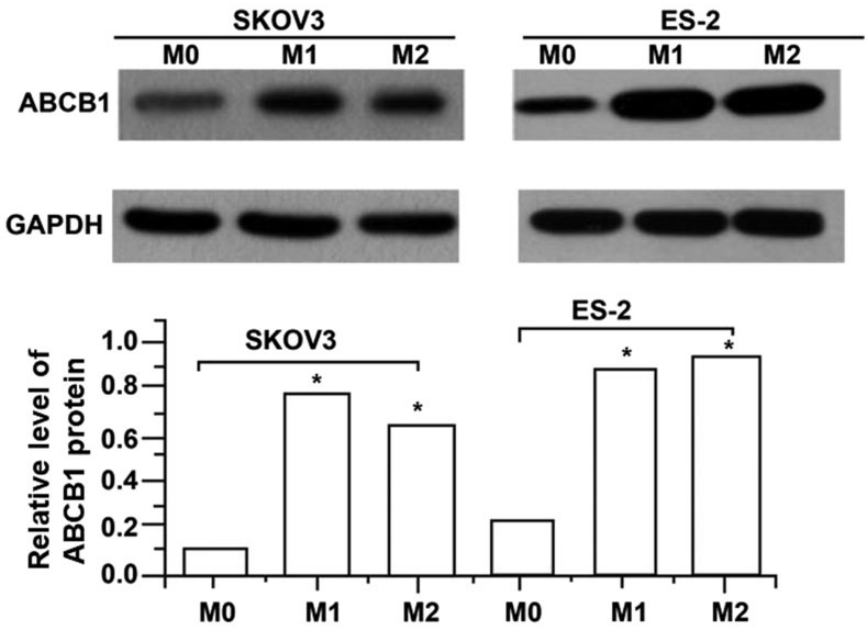

Figure 1. Analysis of P-gp protein expression. SKOV3/ES-2 cells were stably transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids, and P-gp protein levels were detected by western blotting. Representative and quantitative western blot results are shown. M0: control; M1: cells transfected with *ABCB1* 3435 C/wt plasmids; M2: cells transfected with *ABCB1* 3435T/mut plasmids. *$P<0.05$, compared with M0.

Effect of the ABCB1 (3435C > T) polymorphism on drug accumulation

HPLC was used to determine the drug concentration in cells following the application of cyclosporin A. After incubation with various concentrations of cyclosporin A for 30 minutes, docetaxel was added for 4 hours. In the existence of the inhibitor, the intake of docetaxel significantly increased in both control and transfected cells, and drug accumulation was positively correlated with the inhibitor concentration ($P < 0.05$, Figure 4). The intake of docetaxel in control cells was much higher than in stably transfected cells regardless of whether cyclosporin A was added. Moreover, transfection of *ABCB1* 3435T/mut reduced cellular drug accumulation compared with *ABCB1* 3435C/wt. These results indicate that *ABCB1* 3435T increases the drug transport rate mediated by P-gp, and that cyclosporin A reduces this rate in a dose-dependent manner.

Effect of the ABCB1 (3435C > T) polymorphism on drug transmembrane transport

To investigate the transmembrane transport of docetaxel, a bidirectional transfer test was performed. Cells stably transfected with *ABCB1* 3435C/wt or *ABCB1* 3435T/mut showed strong resistance to docetaxel. The permeability ratio of docetaxel in control cells was >1, indicating the drug transport direction. The transmembrane transport of docetaxel in cells stably transfected with *ABCB1* 3435C/wt or *ABCB1* 3435T/mut was significantly increased compared with control cells ($P < 0.05$), suggesting that the overexpression of P-gp

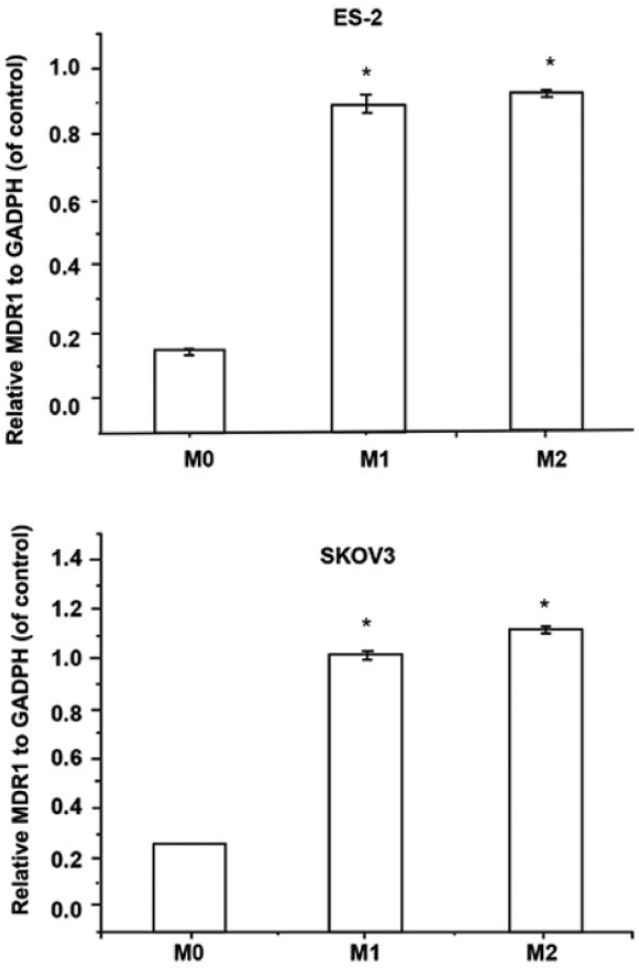

Figure 2. Analysis of *P-gp* mRNA expression. SKOV3/ES-2 cells were stably transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids. *P-gp* mRNA levels were detected by RT-PCR. M0: control; M1: cells transfected with *ABCB1* 3435 C/wt plasmids; M2: cells transfected with *ABCB1* 3435T/mut plasmids. *$P<0.05$, compared with M0.

increased drug transport. Additionally, the relative permeability ratio of docetaxel in cells stably transfected with *ABCB1* 3435T/mut was 1.47-fold higher than in cells stably transfected with *ABCB1* 3435C/wt ($P < 0.05$, Figure 5). Under the effect of cyclosporine A, transmembrane permeability in cells stably transfected with *ABCB1* 3435C/wt or *ABCB1* 3435T/mut decreased significantly ($P < 0.05$, Figure 5). These results indicate that *ABCB1* (3435C > T) SNPs could promote the transport of P-gp-mediated docetaxel.

Effect of the ABCB1 (3435C > T) polymorphism on drug resistance against docetaxel in transplanted tumors

To investigate the effect of *ABCB1* (3435C > T) polymorphisms on tumor drug

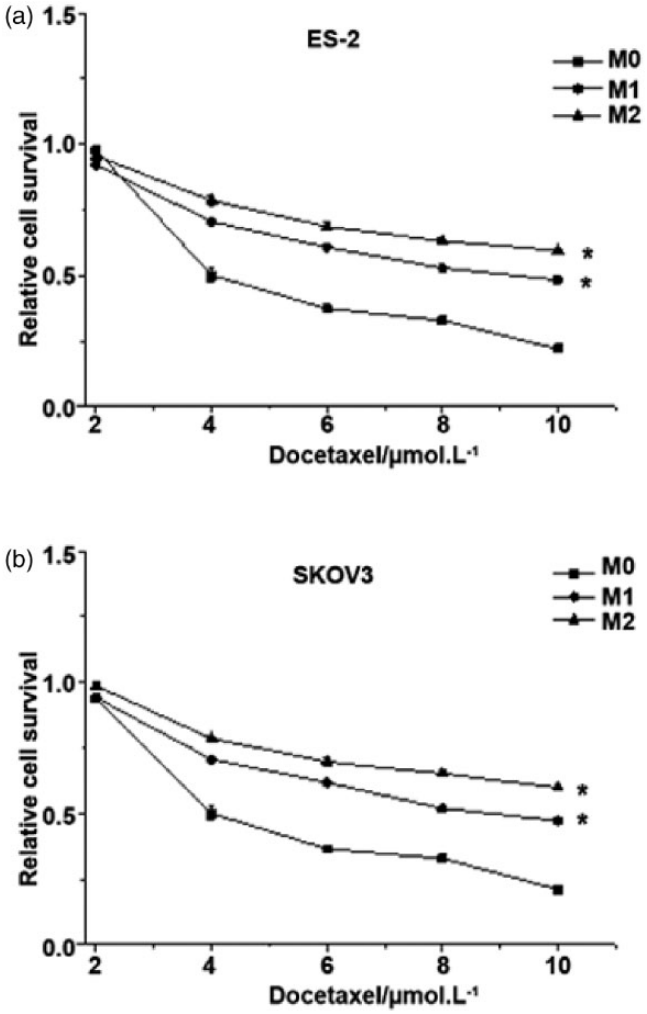

Figure 3. The cytotoxicity of docetaxel on ES-2 and SKOV3 cells. SKOV3/ES-2 cells were transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids. Cell survival was assessed using the MTT assay. M0: control; M1: cells transfected with *ABCB1* 3435 C/wt plasmids; M2: cells transfected with *ABCB1* 3435T/mut plasmids. *$P<0.05$, compared with M0.

sensitivity, a tumor-bearing model was established to determine drug resistance against docetaxel by measuring tumor weight. *ABCB1* (3435C > T) was found to significantly reduce the efficacy of docetaxel. On day 20, the average tumor weight of the control group was 0.4308 g compared with 0.9197 g in the *ABCB1* 3435 C/wt group and 1.9234 g in the *ABCB1* 3435T/mut group ($P < 0.05$, Figure 6). This indicated that *ABCB1* (3435C > T) enhances docetaxel resistance in the ovarian cancer cells of tumor-bearing mice.

Discussion

In 1976, Miano et al.[19] first identified the ABCB1 transporter P-gp in drug-resistant

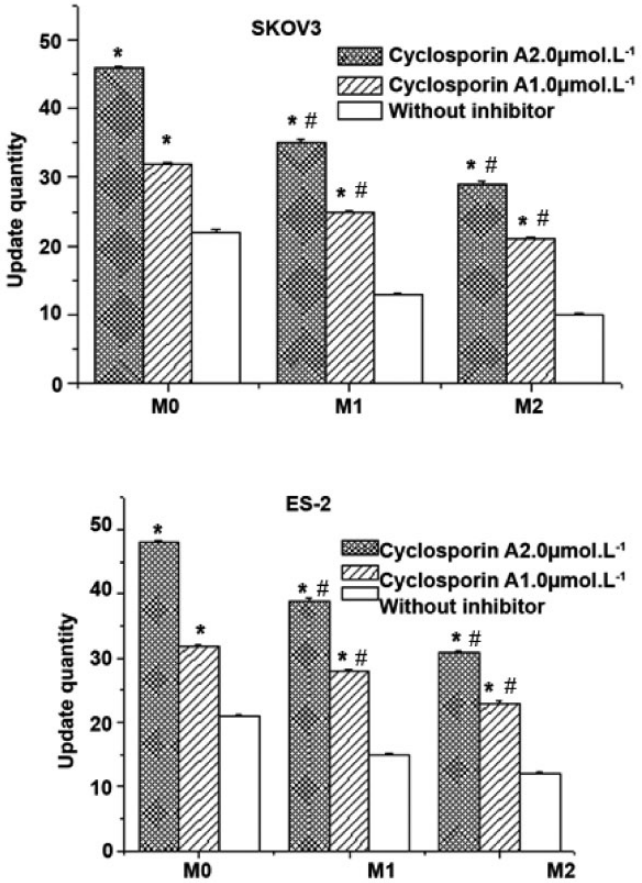

Figure 4. Analysis of docetaxel accumulation in ES-2 and SKOV3 cells. SKOV3/ES-2 cells were transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids. Drug accumulation was assessed in the presence or absence of cyclosporin A. M0: control; M1: cells transfected with *ABCB1* 3435 C/wt plasmids; M2: cells transfected with *ABCB1* 3435T/mut plasmids. *$P<0.05$, compared with the group without inhibitor; #$P<0.05$, compared with M0.

Chinese hamster ovarian cells. Since then, many studies have shown that almost all human tumor cells express *ABCB1* at varying levels. *ABCB1* was found to be primarily expressed in colon cancer, rectal cancer, pancreatic cancer, adrenocortical carcinoma, and liver cancer cells, and to exhibit highly inducible expression in acute leukemia, lymphoma, and ovarian cancer.[20–22] High *ABCB1* expression is usually associated with poor prognosis in cancer patients.[23,24] It affects the absorption, distribution, metabolism, and excretion of drugs[25–27] such as the anticancer medicines colchicine, quinidine, tacrolimus, etoposide, doxorubicin, paclitaxel, and vinblastine.[26] The overexpression of *ABCB1* in cancer cells increases drug efflux and reduces drug concentrations, thus reducing the cytotoxicity of the drug against tumor cells and resulting in drug resistance.

ABCB1 C3435T is located in exon 26 and is a synonymous mutation.[28,29] It does not cause remarkable changes in

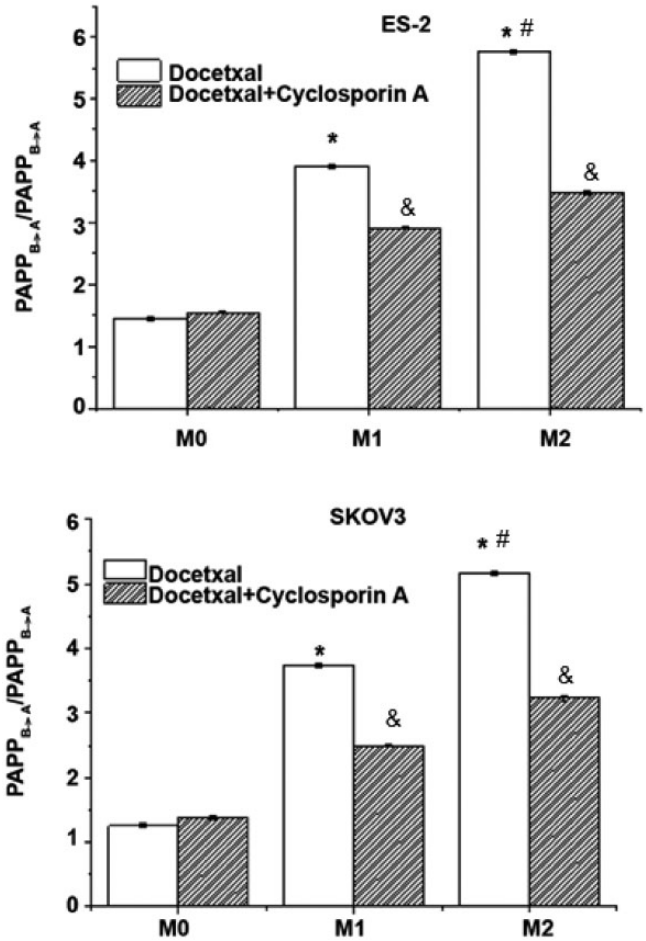

Figure 5. Analysis of docetaxel transmembrane transport in ES-2 and SKOV3 cells. SKOV3/ES-2 cells were transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids and docetaxel transmembrane transport was detected in the presence or absence of cyclosporin A. M0: control; M1: cells transfected with *ABCB1* 3435 C/wt plasmids; M2: cells transfected with *ABCB1* 3435T/mut plasmids. $^{*}P<0.05$, compared with M0; $^{\#}P<0.05$, compared with M1; $^{\&}P<0.05$, compared with docetaxel.

protein expression, but attenuates the efflux function of P-gp by altering the protein conformation.[30] However, research showed that P-gp expression in the renal cortex and duodenum of individuals harboring *ABCB1* 3435TT was much lower than in those with wild-type *ABCB1*, indicating that P-gp protein expression is affected by the *ABCB1* C3435T mutation in some tissues.[31,32] Additionally, many studies reported a strong linkage disequilibrium between *ABCB1* C3435T, G2677T/A, and C1236T, indicating that a specific haplotype synergistically regulates the expression and function of P-gp.[33,34] *ABCB1* (3435C > T) may also be associated with the treatment response and adverse effects of methotrexate,[35] and the metabolism and disposal of substrates by P-gp.[29,36]

In the present study, two stable cell lines (ES-2 and SKOV3) transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids

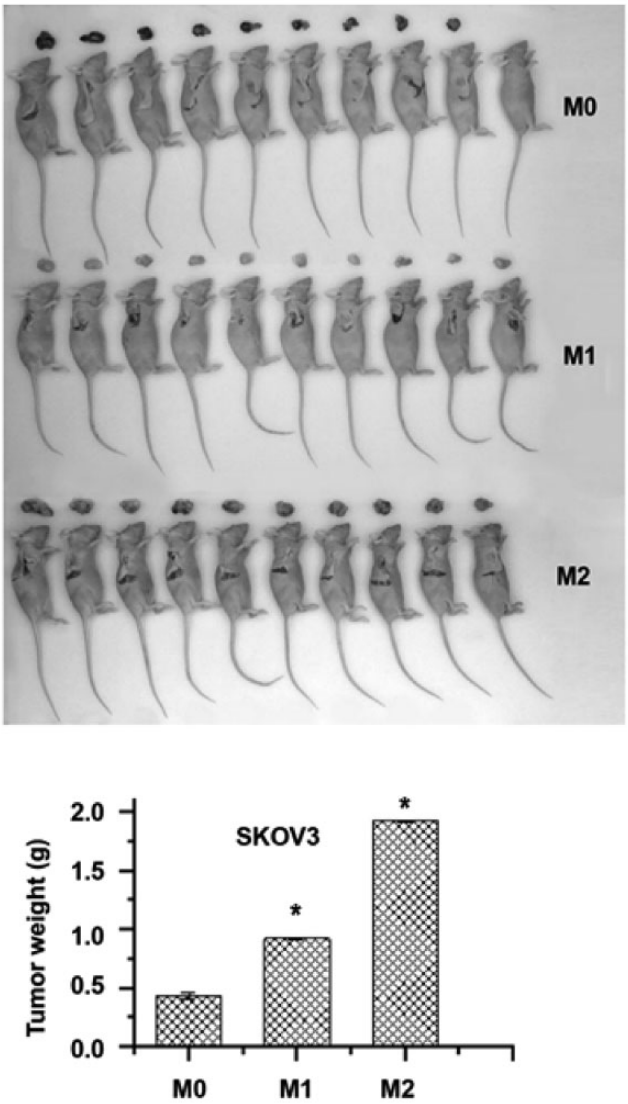

Figure 6. The antitumor effect of docetaxel on mice transplanted with SKOV3 cells stably transfected with *ABCB1* 3435 C/wt or *ABCB1* 3435T/mut plasmids. The effect of docetaxel on transplanted tumors was evaluated by tumor weight. Gross morphology of tumors is shown in the upper panel and tumor weight is shown in the lower panel. M0: control; M1: cells stably transfected with *ABCB1* 3435 C/wt plasmids; M2: cells stably transfected with *ABCB1* 3435T/mut plasmids. *$P<0.05$, compared with M0.

were derived to study the effects of the *ABCB1* (3435C > T) SNP on the transportation of docetaxel as mediated by P-gp. *ABCB1* (3435C > T) plasmid transfection significantly increased the mRNA and protein expression of P-gp. The influence of *ABCB1* SNPs on P-gp expression may involve several mechanisms, although these remain controversial.[29,31,34,35,37] Hoffmeyer et al.[31] first reported that *ABCB1* SNPs

affected the protein expression of P-gp, showing that *ABCB1* (3435C > T) was closely associated with P-gp expression in the human duodenum. It has also been postulated that the effects of c3435T on the P-gp phenotype are mediated not only through changes in amino acid sequences,[36,38,39] but also via epigenetic differences. Further studies are therefore warranted to clarify these mechanisms.

We conducted a docetaxel cytotoxicity test in our stable cell lines, and showed that the drug resistance of transfected cells was higher than that of control cells, while cells transfected with *ABCB1* 3435T/mut demonstrated greater drug resistance than those transfected with *ABCB1* 3435C/wt. Moreover, drug accumulation was higher in control cells than transfected cells, regardless of cyclosporine A addition, and was higher in the *ABCB1* 3435C/wt group than the *ABCB1* 3435T/mut group. This indicated that the *ABCB1* 3435 T/mut allele has a strong efflux effect on docetaxel.

We also found that changes in P-gp activity affected transmembrane transport. P-gp-mediated transport of docetaxel was promoted to a greater extent in cells carrying 3435T/mut than in *ABCB1* 3435 C/wt cells, although the transport rate of control cells was always lower than that of transfected cells in the presence or absence of P-gp inhibitor. This agrees with previous findings that *ABCB1* mutations affect P-gp activity,[29,37] thus altering the clearance of docetaxel. These results together suggest that *ABCB1* over-expression is effective in transporting docetaxel out of cells.

Our *in vivo* experimental results were consistent with the *in vitro* findings in that drug resistance of mice implanted with transfected cells was stronger than in the control group. Mice implanted with *ABCB1* 3435T-containing cells showed a stronger drug resistance to docetaxel, probably because of reduced accumulation leading to decreased drug toxicity.

Conclusion

P-gp encoded by the *ABCB1* variant allele appears to be more efficient at transporting docetaxel compared with the wild-type allele. Thus, the *ABCB1* 3435C > T SNP drastically influences P-gp expression and activity and its role in the efflux of docetaxel. Because the extent of drug resistance will affect the therapeutic effect, patients carrying the *ABCB1* 3435C > T SNP should be administered a combination of multiple drugs to avoid drug resistance.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Ethics approval and consent to participate

Ethical approval was not required for this study.

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