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Original Article

Developing multidrug-resistant cells and exploring correlation between BCRP/ABCG2 over-expression and DNA methyltransferase

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Expression of breast cancer resistance protein/ATP-binding cassette sub-family G member 2 (BCRP/ABCG2) is the major cause of chemotherapy failure. It is important to establish and characterize the multidrug resistance cells and to investigate the mechanism of multidrug resistance. Multidrug-resistant cells expressing BCRP/ABCG2 based on human breast cancer MCF-7/wt cells were developed by gradually increasing application of low concentration of mitoxantrone. Real-time quantitative PCR, western blot, and immunofluorescence assay were employed to analyze BCRP mRNA and protein expression. Drug accumulation in the cells was measured by flow cytometry and DNA methyltransferases were analyzed by western blot. The results indicated that the inhibitory ratio of cell proliferative growth exhibited an exponential relation with the concentration of mitoxantrone. The IC_{50} of MCF-7/wt cells to mitoxantrone was found to be 0.42 μM. 3-(4,5-Dimethylthlthiazol-2-YI)-2,5-Diphenyltetrazolium Bromide assay indicated that the mitoxantrone-resistant cells at different stages exhibited cross-resistance to adriamycin and taxol. BCRP/ABCG2 mRNA and protein levels in the mitoxantrone-resistant cells at different stages increased with increasing concentration of mitoxantrone. Intracellular accumulation of mitoxantrone in the cells decreased with the increase of the BCRP/ABCG2 expression levels. DNA methyltransferase 1 (DNMT1) and DNA methyltransferase 3a (DNMT3a) expressions in the cells at different stages decreased slightly, whereas DNA methyltransferase 3b (DNMT3b) expression decreases significantly. BCRP/ABCG2 overexpression and its drug-efflux function in the drug-resistant cells are the main factors to produce multidrug resistance. Our results suggest that multidrug resistance is related to overexpression of BCRP/ABCG2 and the decrease of DNA methyltransferases, especially DNMT3b.

Keywords breast cancer resistance protein (BCRP); ATP-binding cassette sub-family G member 2 (ABCG2); drug-efflux transporter; multidrug resistance; DNA methyltransferase

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Introduction

The resistance of cells to functionally and structurally unrelated chemotherapeutic agents, named multidrug resistance (MDR), is usually mediated by over-expression of several ATP-binding cassette (ABC) transporters, including ABCB1 (MDR1/Pgp), members of the ABCC transporter (MRP) and ABCG2 (BCRP/MXR) [1,2]. Human breast cancer resistance protein (BCRP/gene symbol ABCG2) was previously isolated from the MCF-7 breast cancer cells which are resistant to doxorubicin. Unlike P-gp and MRP1, BCRP/ABCG2 is composed of 655 amino acids with a molecular mass of 72 kDa and localized at the plasma membrane with only one transmembrane domain with six transmembrane segments and one nucleotide-binding site [2,3]. It is an ABC half-transporter and also belongs to ABC transmembrane transporter family [4,5]. BCRP/ABCG2 expresses physiologically in the placenta, brain microvessel endothelium, mammary gland, bile canaliculi, small intestine, colon, liver, biliary tract, ovary, testis, kidney, and also in hematopoietic stem cells [6–9], which may play a crucial role in protecting the organs from toxic compounds in normal and cancer cells as a drug-'efflux pump'. BCRP/ABCG2 transports a diverse array of substrates, including doxorubicin, mitoxantrone, topotecan, methotrexate, daunorubicin, SN-38, porphyrin, imatinib, and gefitinib, out off cells [10–12]. It causes a decrease of intracellular concentration of chemotherapeutic drugs in BCRP/ABCG2 expressed cells. Although the mechanism of BCRP/ABCG2 as a drug-'efflux pump' has been intensively investigated, there is still much to be elucidated on the molecular mechanism. Current evidence indicates that BCRP/ABCG2 transcription is regulated by hypoxia [13] or

estrogen response elements located in the promoter region of the BCRP gene [14,15]. It is also regulated by the methylation of the promoter region of the BCRP/ABCG2 gene [16–18]. There is much evidence indicating that hypermethylation of tumors is related to DNA methyltransferases (DNMTs). DNMTs are over-expressed in various types of tumor e.g. leukemia, colorectal cancer, prostate cancer, ovarian cancer, endometrial cancer and breast cancer [19–21]. DNMT1 and DNMT3b constitute the major activities, which can be inferred from the strongly reduced DNA methylation levels in DNMT1 and DNMT3b double knockout cell lines. It has also been shown that the inhibition of DNA methyltransferase activity can lead to demethylation and drug resistance [22,23].

Therefore, the main purpose of this study is to examine whether the BCRP/ABCG2 expression is correlated with the concentration of mitoxantrone applied to the cells at different stages, to determine the role of BCRP/ABCG2 in drug-'efflux pump', and to elucidate the molecular mechanisms in up-regulating BCRP/ABCG2 expression and DNA methyltransferases variation in the drug-resistant cells.

Materials and Methods

Cell line and culture

Human breast cancer MCF-7/wt cell line was obtained from the Institute of Biochemistry and Cell Biology, CAS (Shanghai, China). The cells were grown in RPMI-1640 medium (GIBCO, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml ampicillin and streptomycin. The cells were cultured in the humidified 5% CO_2 incubator at 37°C.

Assay of cell sensitivity to chemotherapeutic drugs

MCF-7/wt cells were seeded at a density of 5×10^4 per well in 96-well plates in RPMI-1640 medium containing 10% FBS, 100 U/ml ampicillin, and 100 μg/ml streptomycin, incubated for 24 h. Then the medium were changed to 200 μl of the medium containing mitoxantrone (TUNG CHIT Pharmaceutical Co. Ltd., Nanjing, China) at different concentrations, and continued to incubate at 37°C for 48 h. MTT assay was performed by adding 10 μl of CCK-8 solution (Cell counting kit/CCK-8, Dojindo, Japan) into each well and the cells were further incubated at 37°C for 2 h. The OD_{480} were measured by a microplate reader (Bio-Rad, Hercules, USA). The fractional absorbance was calculated by the following formula:

$$\text{Cellsurvival} = \frac{\text{mean absorbance in testwell}}{\text{mean absorbance in control wells}} \times 100\%.$$

The IC_{50} values were defined as the concentration of drug resulting in 50% survival of the mitoxantrone-treated cells.

Development of mitoxantrone-resistant cells and test of their sensitivity to chemotherapeutic drugs

MCF-7/wt cells were seeded at a density of 5×10^4 per well in 96-well plates in RPMI-1640 medium containing 10% FBS, 100 U/ml ampicillin, and 100 μg/ml streptomycin. We selected 0.01 μM of mitoxantrone as a start, which is lower than 1/10 ID_{50} of MCF-7/wt cells and increased the concentration of mitoxantrone stepwise- to attain drug-resistant cells at different stages. The cells were cultured at least for 1 month at specific mitoxantrone concentrations of 0.01, 0.1, 0.2, 0.4, and 0.8 μM, respectively. Therefore, more than 5 months was needed for attaining mitoxantrone resistance at 0.8 μM. The mitoxantrone-resistant cells were obtained and cultured in the medium containing corresponding concentration of mitoxantrone. The mitoxantrone-resistant cells at different stages were seeded at a density of 5×10^4 per well in 96-well plates to test the sensitivity to the chemotherapeutic agents adriamycin, taxol, and mitoxantrone.

RNA isolation and reverse transcription

Total RNA was extracted from MCF-7/wt cells and drug-resistant cells at different stages by using Trizol reagent (Invitrogen, Carlsbad, USA). Single-strand cDNA was reverse transcribed by using Reverse-transcription kit (TaKaRa, Dalian, China) and an $Oligo(dT)_{18}$ primer according to the manufacturer's protocol. Reverse transcription was performed at 42°C for 60 min using $Oligo(dT)_{18}$ primer and 20 U of AMV reverse transcriptase.

Quantitative real-time PCR

Quantitative real-time PCR was performed with MX4000 multiplex quantitative PCR system (Stratagene, La Jolla, USA). The primers were designed by Primer 3.0 software and then synthesized by TaKaRa Biotechnology Co. Ltd. The sequences of the primers are listed in **Table 1**. Each PCR was carried out in a 25 μl reaction mixture containing 12.5 μl of SYBR premid EX Taq, 1 μl of each primer, 0.5 μl of Rox Reference Dye II, 0.5 μg of cDNA template and ddH_2O (to a final volume of 25 μl). The final concentrations of the primers and Mg^{2+} were 0.5 mM and 4 mM, respectively. The thermal cycling conditions were as follows: 95°C for 10 min, 95°C for 30 s, 60°C for 60 s, and 72°C for 50 s, and 40 cycles for amplifying BCRP and β-actin.

The PCR cycle number that generated the first fluorescent signal above a threshold value [the threshold cycle (C_T)] was determined. The threshold was calculated as a value of 10 SDs above the mean fluorescence generated during the baseline cycles. A comparative C_T method ($2^{-\triangle\triangle C_T}$ method) was used to detect relative gene expression [24]. The following formula was used to calculate the relative amount of the transcript of interest in the

Table 1 Primers used for real-time quantitative PCR to amplify BCRP and β-actin

Primer	Primer sequence	Location on mRNA[a]	Expected base pair (bp)
BCRPRTL	5′-TCCACTGCTGTGGCATTAAA-3′	1142–1161	418
BCRPRTR	5′-TGCTGAAACACTGGTTGGTC-3′	1539–1559	
β-Actin L	5′-TCCCTGGAGAAGAGCTACGA-3′	703–722	309
β-Actin R	5′-GTACTTGCGCTCAGGAGGAG-3′	922–1011	

[a]Accession number AF098951.

treated sample (X) and the control (calibrator) sample (Y), both normalized to an endogenous reference (β-actin) $2^{-\triangle\triangle C_T}$:

$$X = \Delta C_{T,X} - \Delta C_{T,Y},$$

where ΔC_T stands for the difference in C_T between the transcript of interest and β-actin, and the $\Delta\Delta C_T$ for sample untreated MCF-7/wt cells were used as the calibrator for all RT-PCR experiments. The C_T data from duplicate PCRs in which the same cDNA was used were averaged before the $2^{-\triangle\triangle C_T}$ was calculated.

Membrane protein extraction and western blot analysis

Cells of MCF-7/wt and the drug-resistant cells in different stages were cultured in flasks, followed by being washed in D-Hanks. Cell extracts were prepared with 4 ml of cell lysis buffer [0.15 mol/l NaCl, 1% NP-40, 0.01 mol/l deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.05 mol/l Tris–HCl (pH8.0), 1 mmol/l sodium orthovanadate, and 1 mmol/l PMSF] containing protease inhibitor, agitated for 40 min on ice, and then centrifuged for 30 min at 14,000 rpm at 4°C. The insoluble debris was discarded. Protein concentrations were determined using western blotting. Cell extracts (40 μg) were mixed with 5 μl of gel-loading buffer [250 mmol/l Tris–HCl (pH 8.0), 20% mercaptoethanol, 40% glycerol, 8% SDS, 1.2 mg/ml bromophenol blue], heated for 5 min at 95°C and separated on 8% SDS polyacrylamide gels. The human ABCG2 specific mouse monoclonal antibody BXP-21 (Alexis Biochemicals, San Diego, USA), secondary horseradish peroxidase (HRP)-conjugated goat antibody against mouse IgG1 (Sigma, St. Louis, USA), and mouse anti-β-actin (Biovision, Lyon, France) were diluted to 1: 500, 1: 3000, and 1: 1000, respectively. Bands corresponding to total ABCG2 or β-actin were detected by ECL kit (GE Healthcare, Piscataway, USA). The Image J software was employed to analyze the bands.

Flow cytometric detection of BCRP/ABCG2 drug-efflux pump function

Intracellular fluorescence of mitoxantrone was analyzed with an FACScan flow cytometry (Becton Dickinson, Mountain View, USA) equipped with an argon laser using a previously described method with minor modification [25,26]. Briefly, MCF-7/wt cells and the drug-resistant cells at different stages were cultured in 6-well plate at 1×10^5/ml, exposed to 3 μM mitoxantrone, incubated for 2 h bath at 37°C, then washed twice with ice-cold PBS and incubated with water bath at 37°C for another 1 h. The cells were treated with 0.25% trypsin, washed twice with ice-cold PBS, and then suspended in ice-cold PBS. Mitoxantrone-derived fluorescence was measured through a 488 nm band pass filter at an excitation wavelength of 675 nm with an FACscan flowcytometer.

Assay of BCRP/ABCG2 expression by immunofluorescence

An immunofluorescene assay was performed as described previously [27,28]. Briefly, cells were grown on glass coverslips and washed three times with PBS, 10 min each time. Cells were fixed in 4% paraformaldehyde in cold PBS for 30 min, washed three times with PBS, and then incubated in 0.2% Triton X-100/PBS permeabilization for 10 min, followed by washing three times with PBS. Cells were blocked in 5% FBS in PBS for 30 min, washed once with PBS, then incubated with 1: 500 dilution of the human ABCG2 antibody BXP-21 at 4°C overnight. After the cells were washed three times with incubation buffer, the secondary antibody Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) was added at a dilution 1: 1000 and incubated for 30 min at room temperature. Finally, the cells were washed three times with PBS, and the cell nucleoli were stained by DAPI (Applichen, Gatersleben, Germany). The distribution of fluorescence was analyzed with a fluorescence microscope (Carl Zeiss, Goettingen, Germany).

Statistical analysis

Data were presented as mean ± SD of the three independent experiments and were analyzed using one-way ANOVA analysis followed by the paired sample t-test. Differences with $P < 0.05$ or 0.01 were considered as statistically significant.

Results

Development of mitoxantrone-resistant cells

In order to develop the mitoxantrone-resistant cells, we first detected the inhibitory rate of mitoxantrone on MCF-7/wt cell growth and IC_{50}. Different concentrations of mitoxantrone were applied to treat MCF-7/wt cells. The result indicated that the inhibitory ratio of the cell growth showed liner relationship: $Y = 13.5398lgX_0 + 37.6088$. X_0 represents the concentration of mitoxantrone (μg/ml), Y stands for the inhibitory ratio (%), and the relative index R is 0.9911. The IC_{50} is 0.42 μM (Fig. 1). The mitoxantrone-resistant cells, MCF-7/0.05, MCF7/0.1, MCF-7/0.2, MCF-7/0.4 and MCF-7/0.8 were obtained by exposure to stepwise increasing concentrations of mitoxantrone and cultured in medium containing the corresponding concentrations of mitoxantrone. The drug-resistant cells grew without observable change in their proliferation rates in comparison to their parental cells. No obvious morphological change in the drug-resistant cells was observed under an optical microscope.

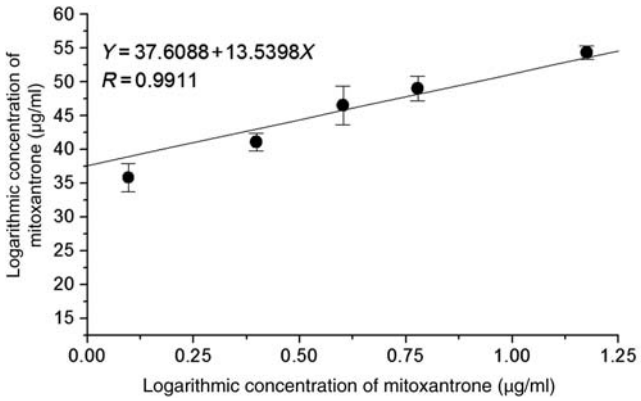

Figure 1 Inhibitory ratio of mitoxantrone on MCF-7/wt cells The mean values in three independent experiments are plotted and bar indicates SD values.

Mitoxantrone-induced cross-resistance to adriamycin and taxol

In order to check whether the mitoxantrone-resistant cells at different stages had a cross-resistance to adriamycin and taxol, MTT assay was applied to analyze the cytotoxicity of different concentrations of adriamycin, taxol, and mitoxantrone. Results indicated that the mitoxantrone-resistant cells at different stages exhibited drug resistance to adriamycin, taxol, and mitoxantrone. The IC_{50} values of the mitoxantrone-resistant cells at different stages to different drugs revealed an increase with increasing concentrations of adriamycin, taxol, and mitoxantrone (Fig. 2). The results suggest that the drug-resistant cells induced by mitoxantrone are also able to resist adriamycin and taxol.

BCRP/ABCG2 mRNA analyzed by real-time quantitative PCR

In order to understand the correlation between the MDR and *BCRP/ABCG2* mRNA expression levels, real-time quantitative PCR was used to analyze the mRNA level in mitoxantrone-resistant cells at different stages. The β-actin was used as a control. In order to prevent the product amplified from the genomic DNA, the primers were designed on 6th and 9th exons, respectively. The amplified PCR product is 428 bp. The result revealed that BCRP/ABCG2 mRNA levels increased in the mitoxantrone resistant cells at different stages with the enhancement of cell tolerance to mitoxantrone (Fig. 3).

Western blot analysis of BCRP/ABCG2 expression in the drug-resistant cells

To test the BCRP/ABCG2 expression in the drug-resistant cells, BCRP/ABCG2 expression was evaluated by western blot. The results indicated that BCRP/ABCG2 expression in the drug-resistant cells at different stages increased with the increasing concentrations of mitoxantrone (Fig. 4). It suggests that drug resistance in the cells is correlated with BCRP/ABCG2 over-expression.

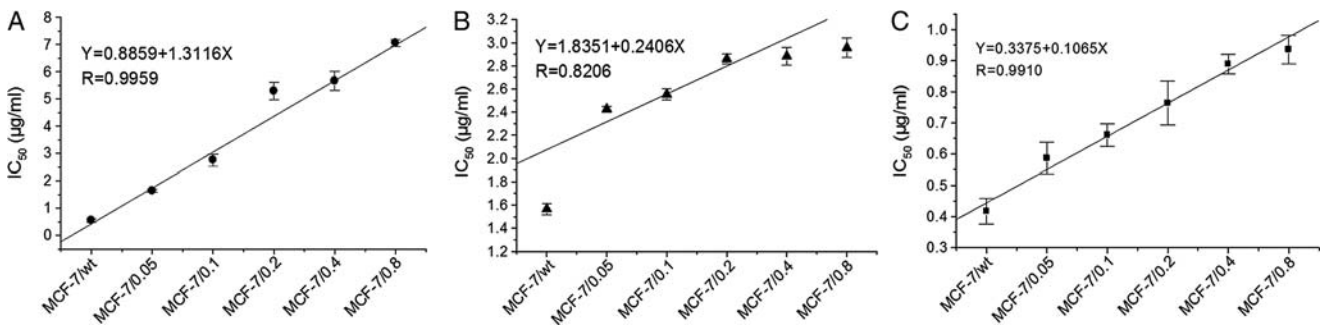

Figure 2 Drug-resistant cells at different stages induced by mitoxantrone are cross-resistant to adriamycin and taxol IC_{50} values of the cells (A) adriamycin; (B) taxol; (C) mitoxantrone.

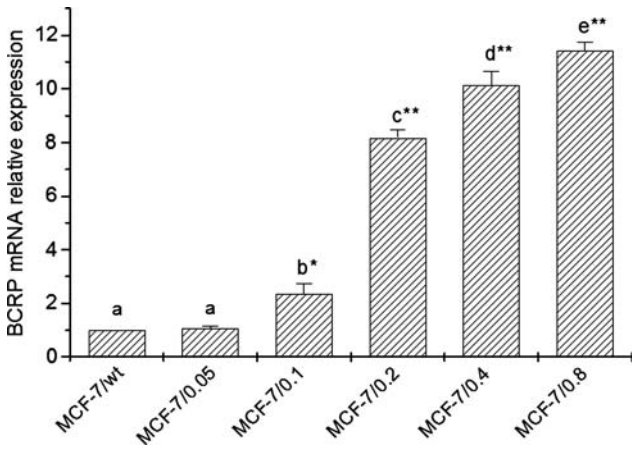

Figure 3 Real-time quantitative PCR analysis of mRNA expression of BCRP/ABCG2 Relative BCRP mRNA expressions in the drug-resistant cells at different stages increased with increasing cell tolerance to mitoxantrone. The mRNA expression was normalized to β-actin expression. The small letters a, b, c, d and e indicate the results of the one-way ANOVA followed by the paired sample *t*-test. The values are arranged in increasing rank order and values bearing the same letter are not statistically different from each other ($n = 3$). The symbols '*' and '**' indicate the significant differences between MCF-7/wt and the different drug-resistant group by paired-sample *t*-test ($^*P < 0.05$ and $^{**}P < 0.01$, respectively).

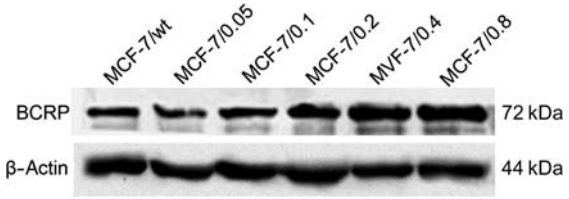

Figure 4 Western blot of BCRP/ABCG2 in MCF-7/wt cells and the drug-resistant cells BCRP/ABCG2 and β-actin protein were detected by western blot. BCRP were detected by the BXP-21 monoclonal antibody diluted to 1: 1000, and secondary HRP-conjugated goat antibody diluted to 1: 3000. The anti-β-actin antibody was diluted to 1: 1000 and the secondary HRP-conjugated rabbit antibody diluted to 1: 2000. Bands corresponding to BCRP and β-actin migrated to approximately 72 and 44 kDa position on 10% SDS-PAGE, respectively.

BCRP/ABCG2 localization in the cells detected by immunofluorescence

Localization of BCRP/ABCG2 in MCF-7/wt cells and the drug-resistant cells at different stages were studied by immunofluorescence analysis. The results revealed that BCRP/ABCG2 was localized at the plasma membrane, as well as in the cytoplasm. In MCF-7/wt cells, it showed the weakly expression, while the expression increased with the cell tolerance to mitoxantrone in the drug-resistant cells at different stages (**Fig. 5**). The results suggest that BCRP/ABCG2 expression is related to the concentration of mitoxantrone.

Intracellular accumulation of mitoxantrone assayed by flow cytometry

To evaluate the BCRP/ABCG2 as a drug-efflux pump conferring the resistance, the intracellular accumulation of mitoxantrone was detected by flow cytometry. The results revealed that the intracellular accumulation of mitoxantrone in the drug-resistant cells at different stages decreased with the BCRP/ABCG2 expression in the drug-resistant cells to mitoxantrone (**Fig. 6**). It suggests that mitoxantrone-induced BCRP/ABCG2 expression in the cells can pump mitoxantrone out of the cells and BCRP/ABCG2 expression is the main mechanism of drug resistance. It also suggests that mitoxantrone is a specific substrate of BCRP/ABCG2.

DNA methyltransferases are involved in the BCRP/ABCG2 expression

In order to clarify the correlation between BCRP/ABCG2 expression and DNA methyltransferase change in MCF-7/wt and the drug-resistant cells at different stages, DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b were analyzed by western blot. The results indicated that the expression levels of the DNMT1 and the DNMT3a in the drug-resistant cells at different stages decrease slightly, whereas the expression level of the DNMT3b in the drug-resistant cells at different stages decreased significantly (**Fig. 7**). These results suggest that BCRP/ABCG2 over-expression is correlated with the decrease of DNA methyltransferase expressions, especially DNMT3b.

Discussion

Drug transporters confer drug resistance during cancer therapy. Among the ABC transporters, BCRP/ABCG2 is involved in drug resistance against anticancer drugs [10,14,29]. In this paper, we reported the development of drug-resistant cells expressing BCRP/ABCG2 in the mitoxantrone-selected cells and detected the BCRP/ABCG2 expression in mRNA and protein levels. The results showed that the levels of BCRP/ABCG2 mRNA expression and protein significantly increased in the mitoxantrone-resistant cells at different stages, they were correlated with the concentration of mitoxantrone applied (**Figs. 3 and 4**). These results are corresponding to the evidence reported previously [30,31]. Current study suggested that over-expression of BCRP in human cancer cell lines resulted in cell resistance to a variety of chemotherapeutic agents, and the functional efflux-pump activity of BCRP almost corresponded to the level of BCRP protein expression [16]. Transfection studies from different laboratories have confirmed that the enforced expression of BCRP/ABCG2 cDNA in different cell types confers the resistance to a variety of chemotherapeutic agents and reduces drug accumulation in the cells [10]. Therefore, BCRP/ABCG2 is a main cause of drug resistance for certain types of chemotherapeutic drugs in tissue culture models. We used adriamycin and taxol to treat the

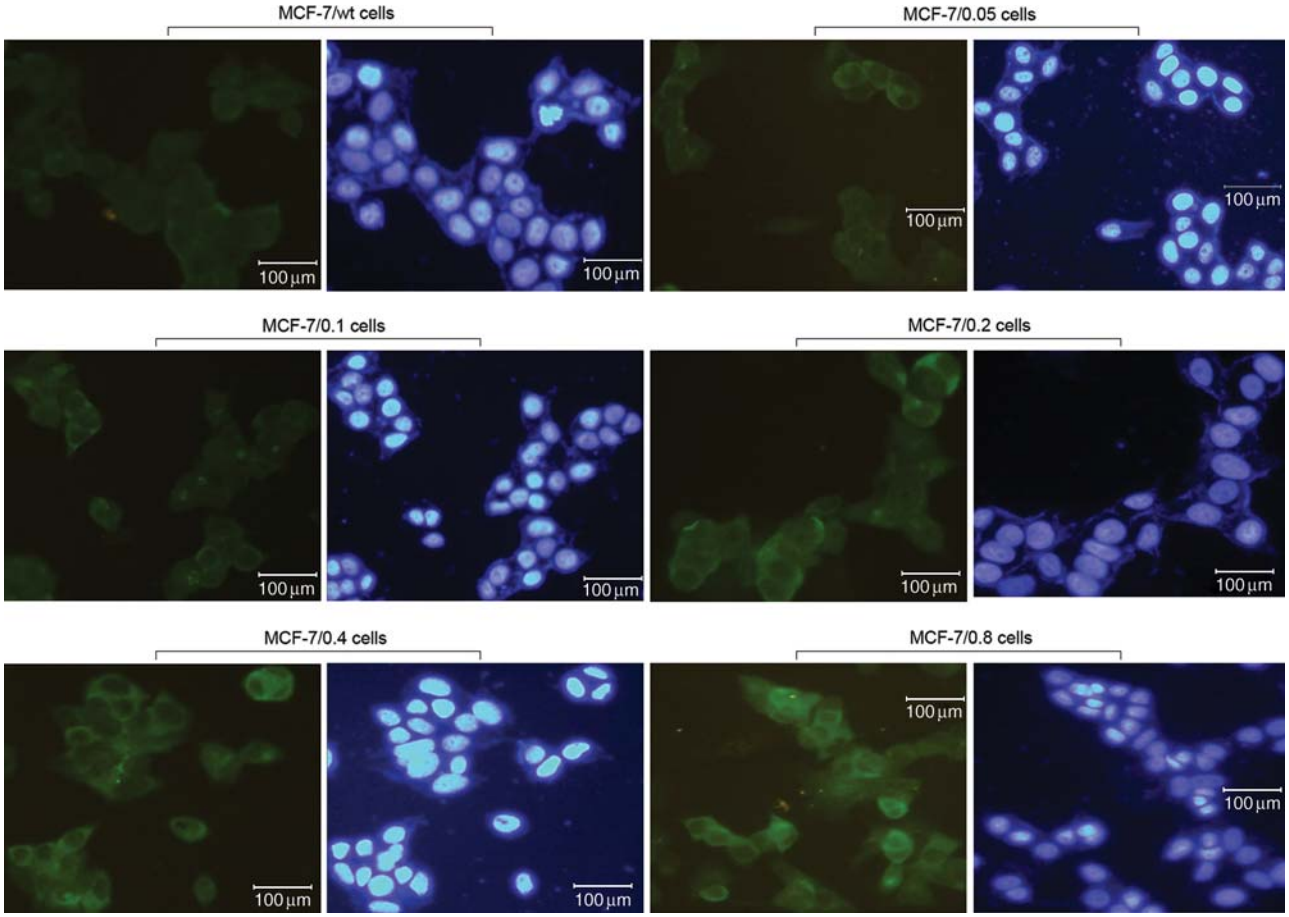

Figure 5 Localization of BCRP/ABCG2 in MCF-7/wt and the drug-resistant cells assayed by immunofluorescence (×400) The left-hand panels show the cells incubated with the BXP-21 monoclonal antibody and goat antimouse IgG secondary antibody (green). Right-hand panels show nuclei of same cells counterstained with DAPI (blue). Green signal represents BCRP/ABCG2 immunofluorescence and blue signal represents nuclei with DAPI.

mitoxantrone-resistant cells and provided the evidence that the IC_{50} values of the mitoxantrone-resistant cells at different stages to adriamycin and taxol increased gradually. Our results also indicated that the mitoxantrone-resistant cells at different stages were also against adriamycin and taxol (**Fig. 2**), which demonstrated that BCRP/ABCG2 might be a multidrug-resistant protein. Some evidence have indicated that the paclitaxel selected cell lines demonstrate a cross-resistance to doxorubicin, vincristine, and mitoxantrane and the doxorubicin-selected cell lines demonstrate a cross-resistance to doxorubicin, vincristine, and mitoxantrane. Doxorubicin-selected cell lines demonstrate a cross-resistance to paclitaxel and vincristine, but not to mitoxantrane [32,33]. We not only observed the drug resistance in the mitoxantrone resistant cells at different stages, but also tested the mRNA by Q-PCR and expressions of BCRP/ABCG2 by western blot. The results revealed that in MCF-7/wt cells BCRP/ABCG2 was expressed extremely low, but they were strongly expressed in the mitoxantrone-resistant cells at different stages. Furthermore, BCRP/ABCG2 specific antibody BXP-21 was used to check the BCRP/ABCG2 localization in the mitoxantrone-resistant cells at different stages. The results showed strong plasma membrane BCRP/ABCG2 localization in the mitoxantrone resistant cells at different stages (**Fig. 6**). In addition, some cytoplasmic staining was also observed with BXP-21 antibody. Surprisingly, MCF-7/wt cell line showed extremely weak immunofluorescence with the BXP-21 antibody, and the results were correlated well with Q-PCR data (**Fig. 3**) and western blot analysis (**Fig. 4**). It was corresponding to the results reported before [34].

The over-expression of BCRP/ABCG2 on plasma membrane protects cells from cytotoxic drugs due to drug efflux, and it is one of the major mechanism responsible for multidrug-resistance. Functional drug efflux by BCRP/ABCG2 is usually detected by flow cytometry [35,36]. To evaluate the role of BCRP/ABCG2 in the mitoxantrone-resistant cells at different stages, we detected the intracellular accumulation of mitoxantrone by flow cytometry. The results indicated that BCRP/ABCG2 levels expressed in mitoxantrone-resistant cells were able to pump mitoxantrone out off the cells. The accumulation of mitoxantrone in the drug-resistant cells at different stages decreased with BCRP/ABCG2 expression compared with MCF-7/wt cells.

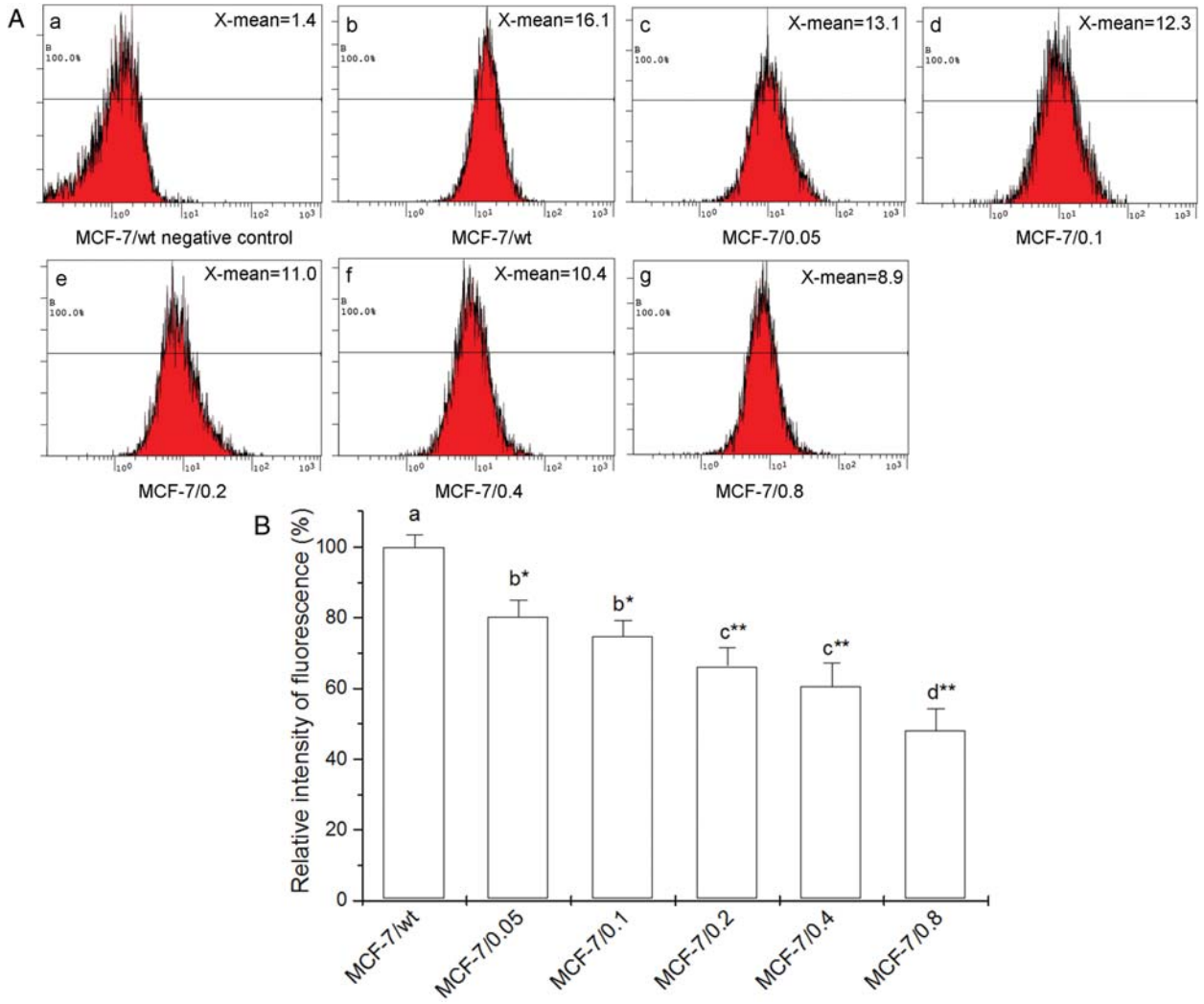

Figure 6 Intracellular mitoxantrone accumulation in the drug-resistant cells assayed by flow cytometry (A) Mitoxantrone accumulation in MCF-7/wt and the drug-resistant cells detected by flow cytometry. (a) MCF-7/wt control; (b) MCF-7/wt positive control; (c) MCF-7/0.05 cells; (d) MCF-7/0.1 cells; (e) MCF-7/0.2 cells; (f) MCF-7/0.4 cells and (g) MCF-7/0.8 cells. Mitoxantrone-derived fluorescence was measured through a 488 nm band pass filter at an excitation wavelength of 675 nm with an FACscan flowcytometer. (B) Correlation between the drug-resistant cells at different stages and intracellular mitoxantrone accumulation in the cells. The mitoxantrone fluorescence was calculated as (the mean fluorescence with mitoxantrone) − (the mean fluorescence without mitoxantrone). The small letters a, b, c and d indicate the results of the one-way ANOVA followed by the paired sample *t*-test. The values are arranged in decreasing rank order and values bearing the same letter are not statistically different from each other ($n = 3$). The symbols '*' and '**' indicate the significant differences between MCF-7/wt and the different drug-resistant group ($^*P < 0.05$ and $^{**}P < 0.01$, respectively).

These data also suggest that BCRP/ABCG2 functions as drug-efflux pump and mitoxantrone is a high-affinity substrate for BCRP/ABCG2.

Currently, the correlation between ABCG2 promoter methylation and BCRP/ABCG2 expression was focused on and investigated [5,16,37]. The results showed that BCRP/ABCG2 expression was inversely correlated with the methylation of ABCG2 promoter that contained a potential CpG island, demethylation of at least one allele is necessary for BCRP re-expression. The percentage of methylated alleles is inversely correlated with ABCG2 mRNA expression, indicating that BCRP/ABCG2 expression was regulated by promoter methylation in both cell lines and patient plasma cells. It has been reported that hypomethylation is associated with MDR1 transcription activation and hypermethylation at the MDR1 promoter CpG islands leads to MDR1 silencing [38]. Some results indicate that the levels of both DNA methylation and histone deacetylation regulate MDR1 gene expression [38,39]. The findings indicate that P-gp over-expression induced by demethylation of the MDR1 promoter is a mechanism of acquisition of drug resistance during chemotherapy. Hypermethylation of the promoter region is related to DNMTs expression in cells. The high expression of DNMTs in cells can enforce

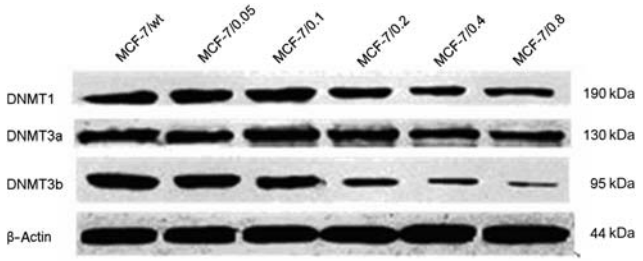

Figure 7 DNA methyltransferases change in the drug-resistant cells SDS-PAGE and western blotting was performed by loading 40 μg proteins per sample onto 10% polyacrylamide gel, and the gel contents transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% nonfat dry milk prepared in TBST, the membrane was probed with a 1: 1000 dilution of rabbit anti-DNMT1, DNMT3a, DNMT3b antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) Membranes were washed with TBST, incubated with the secondary HRP-conjugated goat antibody to rabbit IgG1 was diluted to 1: 3000 and subsequently developed with the enhanced chemiluminescence western blotting kit. The mouse anti-β-actin was diluted to 1: 1000 and was included with the HRP-conjugated rabbit antibody to mouse IgG1 diluted to 1:2000 as the internal loading control.

DNA methylation [40–42]. In this study, in order to clarify the relation between BCRP/ABCG2 expression and DNA methyltransferase, DNMT1, DNMT3a and DNMT3b in the mitoxantrone-resistant cells at different stages were detected by western blot analysis. The results indicated that levels of the DNMT1 and the DNMT3a expression in the mitoxantrone-resistant cells at different stages decreased slightly with BCRP/ABCG2 expression and with resistance to mitoxantrone, whereas the levels of DNMT3b decreased significantly in comparison to DNMT1 and DNMT3a. Yaqinuddin's [17] findings indicated that reduced DNMT3b expression resulted in hypomethylation of retinoblastoma, retinoic-acid receptor β, and adenomatous polyposis coli gene promoters. These results suggest that BCRP/ABCG2 over-expression is related to the hypomethylation of a BCRP promoter region regulated by the decrease of DNA methyltransferase expressions.

In conclusion, BCRP expressions in the mitoxantrone-resistant cells at different stages are mitoxantrone dose-dependent. It functions as a drug-efflux pump to decrease the concentration of mitoxantrone in the cells. BCRP expression is correlated inversely with the levels of DNA methyltransferases, especially with DNMT3b. It will provide insight for epigenetic targeting and antitumor activities in preclinical and early clinical studies.

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