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Reversal of BCRP-Mediated Multidrug Resistance by Stable Expression of Small Interfering RNAs

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Abstract Breast cancer resistance protein (BCRP) is an ATP-binding cassette multidrug transporter that confers resistance to various anticancer drugs like Mitoxantrone. Overexpression of BCRP confers multidrug resistance (MDR) in cancer cells and is a frequent impediment to successful chemotherapy. For stable reversal of BCRP-depending MDR by RNA interference technology, a hU6-RNA gene promoter-driven expression vector encoding anti-BCRP short hairpin RNA (shRNA) molecules was constructed. By treating endogenously and exogenously expresses high levels of BCRP cells with these constructs, expression of the targeted BCRP-encoding mRNA, and transport protein was inhibited completely. Furthermore, the accumulation of mitoxantrone in the anti-BCRP shRNA-treated cells increased. And the sensitivity to mitoxantrone of anti-BCRP shRNA-treated cells is increased 14.6-fold and 2.44-fold respectively compared to their control ($P < 0.05$). These data indicated that stable shRNA-mediated RNAi could be tremendously effective in reversing BCRP-mediated MDR and showed promises in overcoming MDR by gene therapeutic applications. J. Cell. Biochem. 102: 75–81, 2007.

Key words: breast cancer resistance (BCRP); multidrug resistance; RNAi

Tumor is among the top three causes of the death of people. In the treatment of them, chemotherapy has become more and more important and is considered to be a major treatment to avoid the recurrence of cancer after surgery. But we are sorry to find that certain types of cancers, including some of the acute myelogenous leukemia, display intrinsic resistance to multiple chemotherapeutic drugs. Many other cancers acquire multidrug resistance (MDR) [Gottesman et al., 1995] during chemotherapy. This has been a major clinical obstacle for successful treatment of cancer patients. MDR is frequently associated with overexpression of multidrug transporters of the ATP-binding cassette (ABC) superfamily, such as the well-researched member of the ABCA subfamily, P-glycoprotein (P-gp) [Chen et al., 1986] and the MDR-associated proteins (MRP) [Cole et al., 1992], members of ABCC subfamily. A more recently discovered multidrug ABC transporter is the breast cancer resistance protein (BCRP) [Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999], also called ABCG2, ABCP, MXR, a member of the ABCG 'half-transporter' subfamily.

BCRP was first identified in the breast cancer cell line MCF-7/AdrVp, which has MDR phenotype and did not overexpress P-gp or MRP. Different to P-gp or MRP, BCRP have single transmembrane and ATP-binding domains, and functional activity of BCRP argues strongly for activity of the protein as a homodimer [Ozvegy et al., 2001, 2002]. BCRP confers resistance to various anticancer drugs like mitoxantrone (Mit), SN-38, topotecan, and so on. Furthermore, a mutation at position 482 of BCRP may alter substrate specificity of the transporter. Overexpression of the BCRP is found in many cancers [Hoffmann et al., 2001; Kanzaki et al., 2001; Ross et al., 2000]. For example, there is approximately 20–30% in breast cancers [Kanzaki et al., 2001] and about 30–40% in

Abbreviations used: BCRP, breast cancer resistance protein; MDR, multidrug resistance; Mit, mitoxantrone; MRP, multidrug resistance-associated proteins; P-gp, P-glycoprotein; RNAi, RNA interference; shRNA, short hairpin RNA.

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acute leukemia [Ross et al., 2000]. And the expression of BCRP does not correlate either the expression of P-gp or MRP1. This indicated that BCRP can mediate MDR with or without other multidrug transporters. So overcoming BCRP-mediated drug resistance would contribute to cancer chemotherapy [Maliepaard et al., 1999; Kawabata et al., 2001].

A novel means for specific inhibition of a gene is the use of small interfering RNA (siRNA). These 21–23nt long double-stranded RNA (dsRNA) molecules can trigger degradation of eukaryotic mRNA in a sequence-specific manner, an evolutionarily conserved mechanism termed RNA interference [Fire et al., 1998; Elbashir et al., 2001]. More recently, Ee et al. [2004] found that siRNA generated using T7 RNA polymerase downregulated expression of BCRP. However, the exciting silencing efficacy could only be demonstrated in a transient manner. In this study, a construct encoding for a BCRP specific short hairpin RNA (shRNA) was transfected to cells. Results showed that the expression of the endogenous and exogenous BCRP gene all could be inhibited and re-sensitization of drug resistant cells.

MATERIALS AND METHODS

Cell Lines and Culture

JAR (endogenous expression of BCRP highly) cells and MCF-7 human breast carcinoma cells (obtained from Institute of Biochemistry and cell biology, SIBS, CAS) were routinely grown in RPMI-1640 supplemented with 10% heat-inactived FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection and Enforced Expression of BCRP in MCF-7 Cells

The full-length BCRP (wild type) cDNA (pSXL/BCRP was a gift from Dr. Yoshikazu Sugimoto) was inserted into the multiple cloning site of expression vector pCDNA3.1 (Invitrogen) digested by EcoR I. Then pCDNA3.1-BCRP DNA sequence analysis was performed to confirm the insert of the selected clone was in a sense orientation to the cytomegalovirus (CMV) promoter of the pCDNA3.1 vector. MCF-7 cells were transfected with pCDNA3.1-BCRP by using FuGENE6 (Roche Biochemicals) in six-well plates according to the manufacturer's specifications, selected by culture with geneticin (G418, 800 μg/ml). Clones were tested for expression of BCRP by RT-PCR and Western blot analysis. As a control, MCF-7 cells were also transfected with pCDNA3.1 vector and selected by growth in medium containing 800 μg/ml G418.

Construction of Plasmids That Contain DNA Templates for the Synthesis of siRNA Under the Control of the Human U6 Promoter

Plasmid pAVU6+27(gift of Dr. ML He) was used to generate a plasmid that expressing hairpin RNAs. We designed four siRNA sequences according to the cDNA of BCRP. Each anti-BCRP target sequence corresponds to nt 814–832, 874–892, 899–917, 949–967 of BCRP cDNA sequence (GenBank Accession Number: AF098951). They were analyzed by BLAST research to ensure that they did not have significant sequence homology with other genes. We tested the effects of silence with chemical synthesized siRNA (data not shown) and selected the most effective one to construct expression vector as follows: two homologous single-stranded DNA (ssDNA) molecules were chemically synthesized. Annealing of the ssDNA molecules was performed by incubation of 1 μg/μl of each complementary ssDNA oligonucleotide in annealing buffer (0.1 M NaCl, 10 mM tris pH 7.4) in a total volume of 20 μl. The annealing mixture was incubated at 95°C for 5 min followed by cooling slowly to room temperature. The inverted motif that contains the 4-nt spacer and five Ts cloned into the Sal I and Xba I sites of the pAVU6+27 plasmid (Fig. 1). And we also constructed a pAVU6+27/sieGFP in the same way as a control. The ssDNA to aim at the eGFP was chemically synthesized according to Yu et al. [2002].

Transient or Stable Transfection of RNA Interference Plasmid

Firstly, to prove RNA interference phenomena was available in MCF-7 cell line by using pAVU6+27 expression vector, MCF-7 cells were cotransfected with peGFP and pAVU6+27/sieGFP plasmid as previously described and the efficiency of transfection was also evaluated conveniently at the same time. Then MCF-7/BCRP cells and JAR cells were transfected with plasmid pAVU6+27/siBCRP. Also pAVU6+27/sieGFP were used as a control. Cells were harvested 48 or 72 h after transfection. Furthermore, we constructed stable silencing lines. We

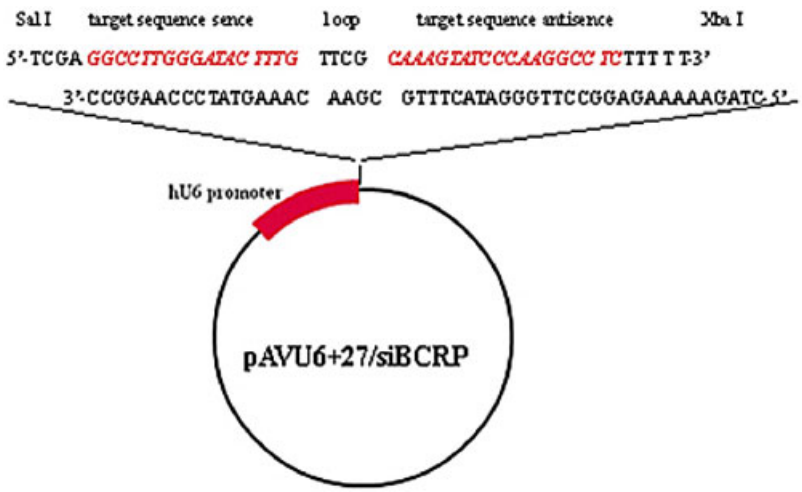

Fig. 1. Construction of anti-BCRP shRNA expression system: sequence of the chemically synthesized shRNA encoding DNA oligonucleotides and schematic structure of the anti-BCRP shRNA expreesion vector pAVU6+27/siBCRP. [Color figure can be viewed in the online issue, which is available at http://www.interscience.wiley.com.]

obtained JAR/Bi cell line by using G418 (500 μg/ml) and MCF-7/BCRP/Bi cell line by hygromycin (400 μg/ml).

Semi-Quantitative Reverse Transcription-PCR

Total RNA was extracted from cells with TRIzol reagent (Invitrogen) and quantified by UV absorbance spectroscopy. The reverse transcription reaction was performed using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas, life sciences). The newly synthesized cDNA was amplified by PCR. The reaction mixture contained 2 μl of cDNA template, 1 μM of BCRP primer (left:5′-tccactgctgtggcattaaa-3′, right:5′-tgctgaaacactggttggtc-3′).

β-actin (left:5′-accgtggagaagagctacga-3′, right: 5′-gtacrrgcgctcagcggag-3′) was used as an internal control. Amplification cycles were 94°C for 5 min, the 25 or 28 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 72°C for 10 min. Aliquots of PCR were electrophoresed on 1.5% agarose gels.

Western Blot Analysis of BCRP

Cells were washed twice with cold PBS and were lysed in ice-cold Tris buffer (50 μM, pH 7.5) containing 5 mM EDTA, 1%SDS, 10 mM PMSF, 0.5 mM DTT for 30 min, sonicated and centrifuged at 12,000*g* for 20 min. The supernatant was used for Western blotting. Thirty micrograms protein were diluted with sample buffer and separated on 5% stacking and 10% resolving SDS–polyacrylamide gels, transferred onto nitrocellulose membranes. To avoid unspecific binding, the filters were incubated in 5% skim milk for 2 h, subsequently, filters were incubated with mouse monoclonal antibodies (BXP-21, ALEXIS Biochemicals) directed against human BCRP diluted in 5% skim milk (1:100) overnight at 4°C, afterwards, with horseradish peroxidase-conjugated goat anti-mouse Ig-G (1:2,000) (Zymed laboratories). The protein-antibody complexes were visualized by chemiluminescent substrate (ECL system, KPL).

Flow Cytometry

The effects of RNAi on intracellular accumulation of mitoxantrone were determined by flow cytometry [Minderman et al., 2002]. Cells (1×10^5) were incubated with 3 μM mitoxantrone for 2 h at 37°C. Then the cells were washed three times with PBS and fresh medium were added for 1 h incubation. The cells were harvested with 0.25% trypsin EDTA and re-suspended in cold PBS before analysis. Measurements of cellular fluorescence were made on a FACSort flow cytometer (Becton–Dickinson) and the emission recorded 675 nm long pass (FL4, mitoxantrone) filters.

Anticancer Drug Sensitivity Assay

Cells were treated with many anticancer drugs. MTT assays were performed as described previously [Kawabata et al., 2001]. The percentage of cell growth was calculated by comparison of the A490 readings from the treated

versus the control. Drug concentration associated with 50% inhibition of growth (IC50) was calculated from linear regression analysis of the linear portion of the growth curves. The difference of cells to anticancer agents was calculated as (IC50 of parental cells)/(IC50 of BCRP-knockdown cells). Each experiment was performed in triplicate at least.

Statistical Analysis

Statistic analyses were carried out using the SPSS 10.0 program for Windows. Student's *t*-test was used to evaluate the statistical significance. A P-value < 0.05 was set as the criteria for statistical significance.

RESULTS

Construction of MCF-7/BCRP Cell Line and Detection the Sensitivity to Anticancer Drugs

pCDNA3.1-BCRP was transfected into MCF-7 cells. MCF-7/BCRP clone was identified by RT-PCR and Western blotting assay. The sensitivity of MCF-7/BCRP cells to Mit, SN-38, DDP, and Taxol were detected by MTT cytotoxicity assay. The results showed that the sensitivity of MCF-7/BCRP to Mit, SN-38 decreased while the sensitivity to DDP and Taxol has no significant difference (data not shown). These data indicated that the MDR breast cancer cell line (MCF-7/BCRP), which exogenously expressed BCRP, was established.

Effects of Hairpin Construct in Transient Transfection

We confirmed the feasibility of our system by using pAVU6+27/sieGFP. And the efficiency of transient transfection was about 30% (data not shown). Then MCF-7/BCRP cells were harvested after transfected with pAVU6+27/siBCRP or pAVU6+27 for 48 and 72 h. RT-PCR was performed and the result showed in Figure 2. We observed that the expression of BCRP in RNA level was decreased when cells transfected with pAVU6+27/siBCRP vector. However, there was no change in the control. This showed that the interfering effect was specific.

Stable Decrease of the BCRP Specific-mRNA and Protein Expression by shRNAs

MCF-7/BCRP cells and JAR cells were transfected with pAVU6+27/siBCRP or control vector. Stable clones were obtained from each transfection after 3 weeks. As shown in Figure 3, the expression levels of mRNA and protein were detected by RT-PCR and Western blot. The decreases were obvious in MCF-7/BCRP/Bi and JAR/Bi cell lines, but no change in cells transfected with control vector.

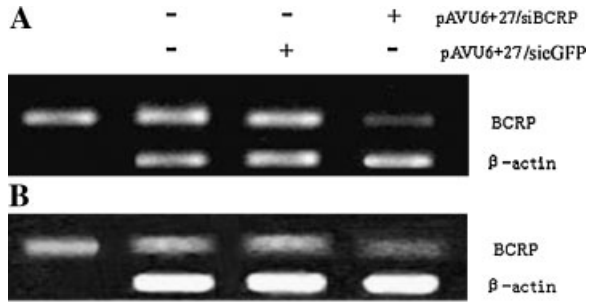

Fig. 2. The expression of BCRP mRNA of each cell was defined by semi-quantitative RT-PCR after transfection 48 h (**A**) and 72 h (**B**). Expression of BCRP and β-actin mRNA was analyzed simultaneously. A marked decrease in the amount of BCRP mRNA when transfected with pAVU6+27/siBCRP. Note that the level of β-actin mRNA expression remains very similar in all three groups.

Increase of Cellular Mitoxantrone Accumulation by shRNA

Effects of RNAi on cellular accumulation of mitoxantrone were investigated by Flow

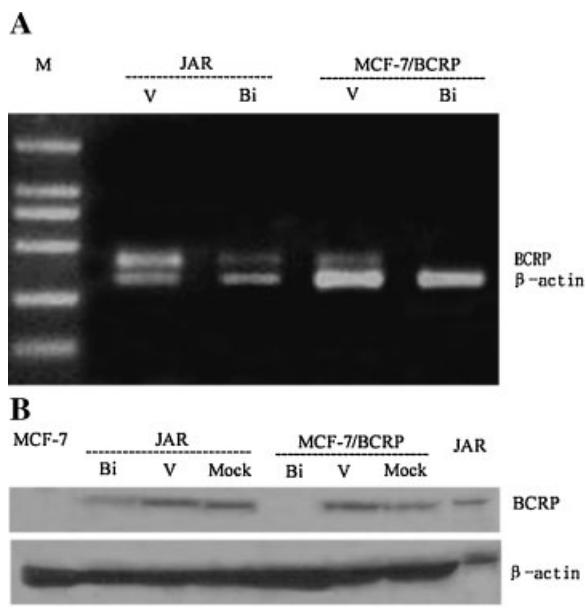

Fig. 3. Effects of shRNA on BCRP expression in JAR cell and MCF-7/BCRP: the level of mRNA (**A**) and protein (**B**) expression of BCRP is decreased in two cell lines which were treated with shRNA (Bi). No change was obtained in the groups which were treated with vector (V) and control (Mock). β-actin expression was analyzed as a loading control.

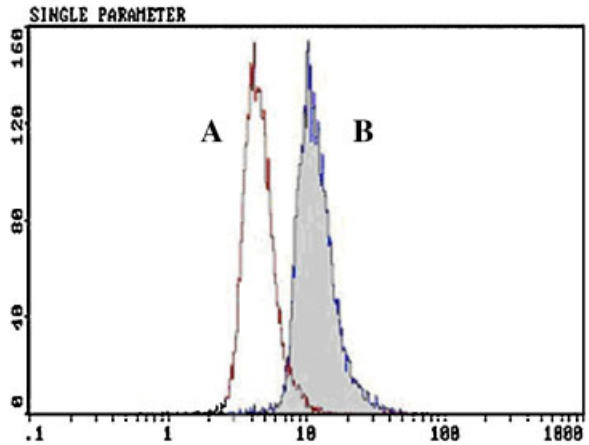

Fig. 4. Effect of shRNA on drug accumulation. Cells were incubated with 3 μM mitoxantrone for 2 h at 37°C. The fluorescence peak shift of the A (unshaded area) to the B (shaded area) indicates increased accumulation of mitoxantrone. A: MCF-7/BCRP/V cell; B: MCF-7/BCRP/Bi cell. [Color figure can be viewed in the online issue, which is available at http://www.interscience.wiley.com.]

cytometry. The result revealed that the median fluorescence value for the MCF-7/BCRP/Bi cell line increased compared with the MCF-7/BCRP/V cell line. Figure 4 indicated that the accumulation of mitoxantrone was increased in MCF-7/BCRP/Bi cell.

Reversal of the Drug-Resistant Phenotype by shRNA

The shRNA-mediated reversal of the multidrug-resistant phenotype was assessed by comparison of the cell survival assay in shRNA-treated cells and control. MCF-7/BCRP/Bi and JAR/Bi cell showed a slightly enhanced chemosensitizing activity compared to their parental cells (Fig. 5). The IC_{50} of shRNA-treated cells to mitoxantrone decreased 14.6-fold and 2.24-fold, respectively ($P < 0.05$).

DISCUSSION

There is considerable evidence that BCRP has a role in clinical MDR [Ross et al., 2000; Hoffmann et al., 2001]. This research also confirmed the role of BCRP in MDR in human breast cancer cell line. It was suggested that chemotherapeutics which cannot be pumped out by BCRP should be utilized when the protein was found highly expressed in some breast cancer patients. In order to guide therapy, it is useful to detect the expression of BCRP in some cancer patients. On the other hand, successful reversal of a multidrug-resistant phenotype would be the basis of an effective drug-based therapeutic strategy. Unfortunately, drugs that modulate classical MDR, such as P-gp inhibitors verapamil, calmodulin antagonists, and cyclosporins, do not reverse the BCRP-mediated atypical MDR. Also there are some identified inhibitors of BCRP activity, such as fumitremorgin C (FTC), GF120918. But their inhibitory effects are not satisfactory. So it is necessary to develop less toxic and more efficient strategies to overcome MDR mediated by BCRP.

Because Elbashir et al. [2001] reported that RNA interference can be triggered in mammalian cells by introduction of 21-nt siRNA. siRNA

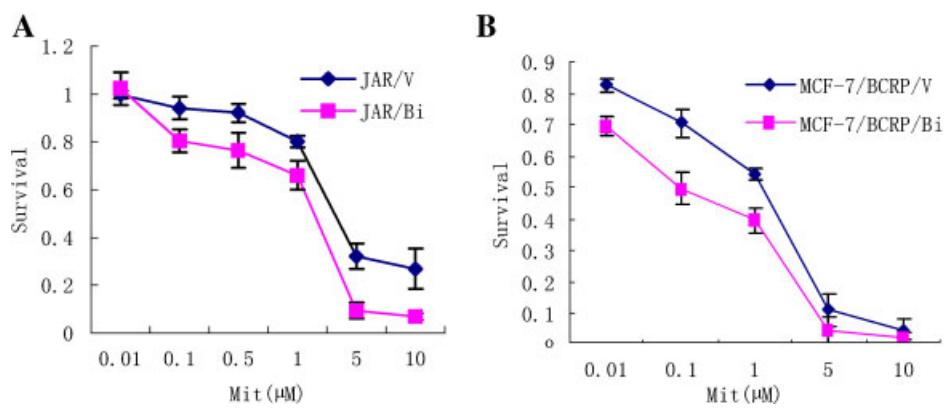

Fig. 5. Effects of shRNA on the sensitivity of JAR cells (**A**) and MCF-7/BCRP cells (**B**) to mitoxantrone. MTT cytotoxicity assay was performed as described in ''Materials and Methods.'' The sensitivity of JAR/Bi to mitoxantrone is increased 2.24-fold. The sensitivity of MCF-7/BCRP/Bi cells to mitoxantrone is increased 14.6-fold. [Color figure can be viewed in the online issue, which is available at http://www.interscience.wiley.com.]

has been shown to be effective approach for silencing gene expression, which has been applied recently to inhibiting MDR1 [Chen et al., 1986; Stege et al., 2004; YagÜe et al., 2004]. And siRNA generated by T7 RNA polymerase to downregulated expression of BCRP was used by Ee et al. [2004]. In this study, the author found another effective siRNA sequence to silence BCRP. Furthermore, a shRNA-expressing vector (pAVU6+27/siBCRP) was constructed to direct the synthesis of shRNAs in cells. In the transient transfection experiment, the decrease of BCRP was detected both in 48 and 72 h (Fig. 2). But neither of them completely inhibited the expression of BCRP. This may depend on the efficiency of transfection and the half-life of the BCRP. The results were satisfactory by stable transfection in this study. Using shRNA decreased the expression of BCRP (Fig. 3), increased intracellular drug accumulation (Fig. 4), and restored drug sensitivity in drug resistant cells. The resistance of mitoxantrone was almost completely reversed (Fig. 5). It was obvious that transient application of chemically synthesized siRNAs was restricted by low transfection efficiencies and short-term cellular persistence of the siRNA molecules [Nieth et al., 2003; Wu et al., 2003]. When using constructed shRNA-expressing vector, the expression of BCRP gene was successfully and completely silenced, and the effect was special. So transfection with shRNA expression vector has more advantage to compare with transfection of 21nt dsRNA homologous which effect is modest in degree and transient in duration. In this way we can go on much longer study, get much stable results because that the inhibition is persistent and stable. On the other hand, it is economical. This study presented the evidence for silencing BCRP gene by shRNA in multidrug-resistant cancer cells. The data demonstrated the proof of principle that endogenous siRNA can be applied to activate RNAi-mediated degradation of the BCRP mRNA in targeted cancer cells.

CONCLUSION

In summary, our study demonstrated the effectiveness of shRNA reversing BCRP-mediated multidrug resistance. The RNA interference approach may be promising for the treatment of drug-resistant cancer. However, consideration of putative physiological role of BCRP, greater control over the timing and localization of the silencing effect would be useful. The development of promoters that express shRNA only in response to small-molecule inducers or in specific tissue types would expand the range of potential experiments.

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