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LIVER CANCER

Reversing multidrug resistance by RNA interference through the suppression of MDR1 gene in human hepatoma cells

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Abstract

AIM: To reverse the multidrug resistance (MDR) by RNA interference (RNAi)-mediated MDR1 suppression in hepatoma cells.

METHODS: For reversing MDR by RNAi technology, two different short hairpin RNAs (shRNAs) were designed and constructed into pGenSil-1 plasmid, respectively. They were then transfected into a highly adriamycin-resistant HepG2 hepatoma cell line (HepG2/ADM). The RNAi effect on MDR was evaluated by real-time PCR, cell cytotoxicity assay and rhodamine 123 (Rh123) efflux assy.

RESULTS: The stably-transfected clones showed various degrees of reversal of MDR phenotype. Surprisingly, the MDR phenotype was completely reversed in two transfected clones.

CONCLUSION: MDR can be reversed by the shRNA-mediated MDRI suppression in HepG2/ADM cells, which provides a valuable clue to make multidrug-resistant hepatoma cells sensitive to anti-cancer drugs.

Key words: Multidrug resistance; ShRNA; MDR1; Hepatocellular carcinoma

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INTRODUCTION

Human hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, especially in Asia and Africa, with an estimated 0.5 million new cases and around 1 million deaths annually[1]. MDR is the most common impediment to successful chemotherapy for a variety of cancers[2], especially HCC. Classic MDR characterized by cross resistance to antineoplastic drugs, is caused by over-expression of MDR1 gene encoding P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter superfamily. In liver, the expression of P-gp on the apical side of various cell membranes enhances the secretion of drugs into bile[3]. A disruption of P-gp-mediated drug extrusion results in a resensitization of tumor cells to treatment with antineoplastic agents, and may allow a successful drug treatment of the multidrug resistant cancer cells.

Most efforts at reversing MDR during the past two decades have been focused on compounds that modulate p-gp activity. In clinical trails, the efficiency of these drugs are difficult to access, mainly because of inherent adverse pharmacokinetic side effects[2], such as hypotension, heart failure, hyperbilirubinemia, and immunosuppression by cyclosporin A. Moreover, tumor cells can acquire resistance to the applied chemosensitizers, a so-called tertiary resistance. Consequently, it is necessary to develop alternative, less toxic and more efficient strategies to overcome MDR. It may, therefore, be more appropriate to target MDR1 gene expression. Indeed, MDR1 transcription has been targeted with ecteinascidin 743 in pre-clinical studies[4] and more recently by modulation of the orphan nuclear receptor SXR[5]. Strategies involving antisense and transcriptional decoy[6] and the use of anti-MDR1 mRNA hammerhead ribozymes have also been suggested [7].

RNAi is a conserved cellular mechanism by which double-stranded RNA (dsRNA) silences the corresponding homologous gene[8]. These 21-25 nucleotides with long, double-stranded small interfering RNA (siRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. Two major discoveries have highlighted the potential of RNAi application in gene therapy. The introduction of synthetic siRNA of 21 nucleotides into mammalian cells can result in efficient gene silencing[9], and shRNAs expressed from RNA promoters within expression vectors also trigger RNAi[10, 11]. Recently, modulation of MDR by transfection of synthetic siRNAs has been reported [12, 13]. However, complete reversal to the drug-sensitive phenotype of parental cells has not been

obtained in any study. This is probably due to two reasons. Firstly, the transfection of synthetic siRNAs causes only transient suppression of target genes, which is often limited to the cell lines that are easy to be transfected. Secondly, the P-gp has a long half life time[14]. Thus, for achieving stable long-term RNAi effects, shRNA is developed[10].

In this study, we constructed two shRNAs targeting different coding regions of human MDR1 gene, because not all siRNA target sequences are equally potent[15]. After two shRNAs were respectively introduced into a multidrug resistant cell line HepG2/ADM derived from HepG2, efficiency of MDR1 gene suppression and whether MDR was reversed were evaluated.

Table 1 Sequences of shRNA oligonucloetides

Oligonucloetides	Sense + loop + antisense DNA template
MDR-A	5′-GATCCGCTTTGGCTGCCATCATCCAttcaagacgTGGATGATGG CAGCCAAAGTTTTTTGTCGACA-3′; 3′-GCGAAACCGACGGTAGTAGGTaagttctgcACCTACTACCGT CGGTTTCAAAAAACAGCTGTTCGA-5′
MDR-B	5′-GATCCGGCCTAATGCCGAACACATttcaagacgATGTGTTCGGCATTAGG CCTTTTTTGTCGACA-3′; 3′-GCCGGATTACGGCTTGTGTAaagttctgcTACACAAGCCGTAATCCGGAA AAAACAGCTGTTCGA-5′

The underlined parts are loops of shRNA oligonucleotides.

MATERIALS AND METHODS

Cell lines and cell culture

The HepG2 cells (Chinese Center for Type Culture Collection) were cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA) contianing 10% of heat-inactivated FCS (Gibco BRL, Gaithersburg, Md. USA). Cells were incubated at 37 ℃ in a 50 mL/L CO_2 air incubator with saturated humidity. The establishment of a multidrug-resistant cell line HepG2/ADM was described previously[16]. The HepG2/ADM cells could survive a 24 h-treatment with adriamycin at a concentration of 5.0 mg/L, and are able to keep growing in the medium containing 1.0 mg/L adriamycin. To maintain MDR phenotype, HepG2/ADM cells were cultured with DMEM supplemented with adriamycin at 1.0 mg/L (Pharmacia, Uppsala, Sweden).

Design of siRNAs and cloning of siRNA hairpin loops

Two different siRNAs targeting different parts of the MDR1 gene (accession number: M14758) were designed: MDR-A (5'-AACTTTGGCTGCCATCATCCA-3', targeting nucleotide 586-606 of MDR1 mRNA sequence) was described previously [17]; MDR-B (5'- AAGGCCTA-ATGCCGAACACAT-3', targeting nucleotide 3494-3514 of MDR1 mRNA sequence) was designed according to the recommendation from the website http://sirna.qiagen.com. The two sequences were individually incorporated into a pair of oligonucleotides (produced by Wuhan GenSil Biotechnology, China. Table 1) where the target sequence appears as antisense followed by sense orientations separated by a 9-nucloetide spacer sequence and flanked at either end by Hind III or BamH1 restriction enzyme sites. The annealed oligonucleotides were cloned into pGenSil-l (Wuhan GenSil Biotechnology, China) according to the manufacturer's recommendations. The transcribed shRNA of MDR-A was 5'ATCCAttcaagacgTG-GATGATGGCAGCCAAAGU- -U-3', and the transcribed shRNA of MDR-B was 5'-GGCCTAATGCCGAACA-CATttcaagacgAT- -GTGTTCGGCATTAGGCCUU-3'. The underlined parts were loops of the two shRNAs.

Transfection and selection of stably transfected cell clones

The HepG2/ADM cells were transfected with 2μg of pGenSil-1/MDR-A, pGenSil-1/MDR-B, or empty vector pGenSil-1. Transfection was performed in 50%-60% confluent cells in 6-well plates using 9 μL of Lipofectamine 2000™ (Invitrogen, USA). Forty-eight hours after transfection, cells were grown in 10 cm cell plates with medium containing G418 (400 μg/mL) but without adriamycin. After 3 wk of culture, visible colonies were picked up and expanded. The stably transfected clone cells were observed to show green fluorescence under microscope and the clones without expression of the transfected gene did not show green fluorescence (Figure 1).

Detection of P-gp Function

Rh123 accumulation is sensitive and specific for indicating the transport function of P-gp[18, 19]. To observe the RNAi efficiency in the transfected cells, Rh123 (Sigma, MO, USA) was chosen to detect P-gp functions. The assay was performed as described by Broxterman *et al* [18]. Briefly, cells were cultured in 6-well plates. When the cells reached 70%-80% confluence, Rh123 was added to cells at a final concentration of 0.25 μg/mL and incubated at 37 ℃ for 1 h. The cells were washed 3 times with 4 ℃ PBS and resuspended at $5\text{-}10 \times 10^5$ cells/mL in 4 ℃ PBS. Rh123 fluorescence was analyzed with a FACStar flow cytometer (Becton Dickinson Immune Cytometry Systems) equipped with an argon laser. The blast population was gated by forward- and side-scatter characteristics. Rh123 fluorescence of 10 000 cells was measured logarithmically through a 530 nm bandpass filter at an excitation wavelength of 488 nm. HepG2 cells without incubation with Rh123 served as a negative control. Rh123 efflux was measured by counting cells in the M1 region of the plot and calculated as percentages of cells in the M1 region of the plot. The bigger percentage of cells in M1 region indicated the greater cellular Rh123 efflux and also a higher P-gp function. All 146 clones were checked.

Real-time reverse transcription chain reaction

Human MDR1 real-time PCR primers used in experiments are 5R-TGGTTCAGG TGGCTCTGGAT-3R and 5R-CTGTAGACAAACGATGAGCTATCA -CA-3R. Human GAPDH primers for real-time PCR used have been previously described[20]. Total RNA was isolated from the cells by TRIzol (Invitrogen, USA) 60-70 d after cell transfection. All RNA samples were stored at -80 ℃ until use. RNAs were reversely transcribed into cDNAs using RevertAid-TM first strand cDNA synthesis kit (Fermentas, Inc.) according to the manufacturer's instructions. Real-time PCR and data collection and result analysis were performed on

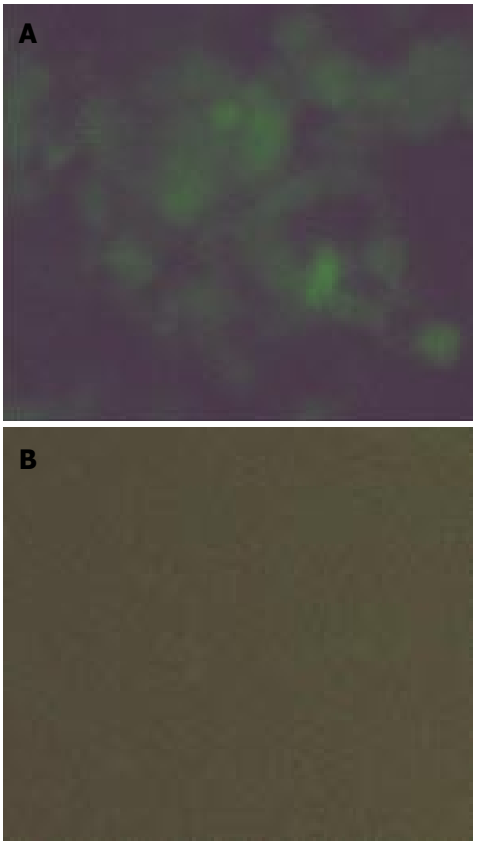

Figure 1 Stably transfected cells showing green fluorescence under a fluorescence microscope (**A**) and cells without stable expression of the transfected gene (**B**).

ABI PRISM®7000 sequence detection system (Applied Biosystem, USA). All quantitations were normalized to an endogenous control GAPDH. The relative quantitation value of each target gene was analyzed using a comparative C_T method. The following formula was used to calculate the relative amount of the transcript in the sample and normalized to an endogenous reference (GAPDH): $2^{-\triangle\triangle CT}$, where $\triangle C_T$ is the difference in C_T between the gene of interest and GAPDH, and $\triangle\triangle C_T$ for the sample = $\triangle C_T$ of the actual sample -$\triangle C_T$ of the lowest expressing sample (used as calibrator). The MDR1/GAPDH ratio in drug sensitive HepG2 cells was set at 1, and $L0g_{10}1 = 0.00$.

Cell cytotoxicity assay

The cytotoxicity assay and ID50 determination of anticancer drugs on the different clone cells were analyzed by the microculture methyl tetrazolium (MTT) method as described previously [21]. Briefly, in each experiment 10 000 cells per well were seeded in 96-well plates and cultured with DMEM or DMEM complemented with adriamycin in dilution series for 48h. Four repeated wells were used for each concentration. The total medium volume of each well was 200 μL. After 48 h of culture, 50 μL of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, MO, USA) at 2 mg/mL was added to each well and incubated for 4 h at 37 ℃. Plates were centrifuged at 800r/min for 6 min, the medium was aspirated from plates, leaving about 30 μL of medium in each well. One hundred and fifty μL of dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany) was added to each well and the plates were shaken for 10 min to solubilize the formazan crystals. The plates were read immediately at 540 nm on a scanning multi-well spectrophotometer (Titertek, Alabama, USA). Absorbance from the cells treated with adriamycin was corrected against the absorbance from the untreated control cells. The ID50 value was defined as the dosage of drugs at which 50% of cells died after 48 h of treatment, which was calculated from 3 independent experiments for each clone. Since the basal growth rates of the cell lines had no significant difference in the first 2 d (data not shown), the errors caused by that were ignored.

Statistical analysis

For statistical analysis of P-gp function and cell cytotoxicity assay, the unpaired *t*-test was performed on SPSS 11.0 software. The real-time PCR data were analyzed using the SDS software on the ABI PRISM®7000 sequence detection system, the confidence limit was set at 95%.

RESULTS

ShRNAs decreased P-gp function

The RNAi efficiency was evaluated by measuring specific P-gp activity. P-gp-mediated transport indicated by intracellular decrease of Rh123 fluorescence was studied using flow cytometry. The P-gp activities of 58 pGenSil-1/MDR-A-transfected clones and 78 pGenSil-1/MDR-B-transfected clones were analyzed. Meanwhile, 10 HepG2/ADM control clones transfected with an empty pGenSil-1 vector were also analyzed. Two of the pGenSil-1/MDR-B-transfected clones showed a complete suppression of the P-gp function. More than 60% of the pGenSil-1/MDR-B-transfected clones (49 of 78 clones) showed an intermediate decrease of the P-gp function, whereas 8 clones did not show any significant changes of P-gp function. None of the pGenSil-1/MDR-A-transfected clones showed a complete suppression of P-gp activity, although different suppressions of P-gp function were detected. None of the control clones exhibited any significant alterations of P-gp function. Compared with HepG2 cells, the P-gp functions of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells were at a low level near to that of the HepG2 cells, the P-gp functions of the HepG2/ADM cells and control HepG2/ADM/vec clone cells were much higher (Figure 2).

ShRNAs decreased MDR1 mRNA level

To further confirm the data from P-gp function study with Rh123 fluorescence, real-time RT-PCR was performed to quantify the RNAi-mediated suppression of MDR1 mRNA. The MDR1 mRNA expressions of parental cells, HepG2/ADM cells, HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells and the HepG2/ADM/vec control clone cells were measured. Expression of MDR-B shRNA in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 reduced MDR1 mRNA expressions by 93% and 92%, respectively. MDR1 mRNA expression was reduced by 40% in the HepG2/ADM/vec control clone (Figure 3).

ShRNAs reversed multidurg-resistant phenotype

The reversal of MDR-mediated by shRNA was measured

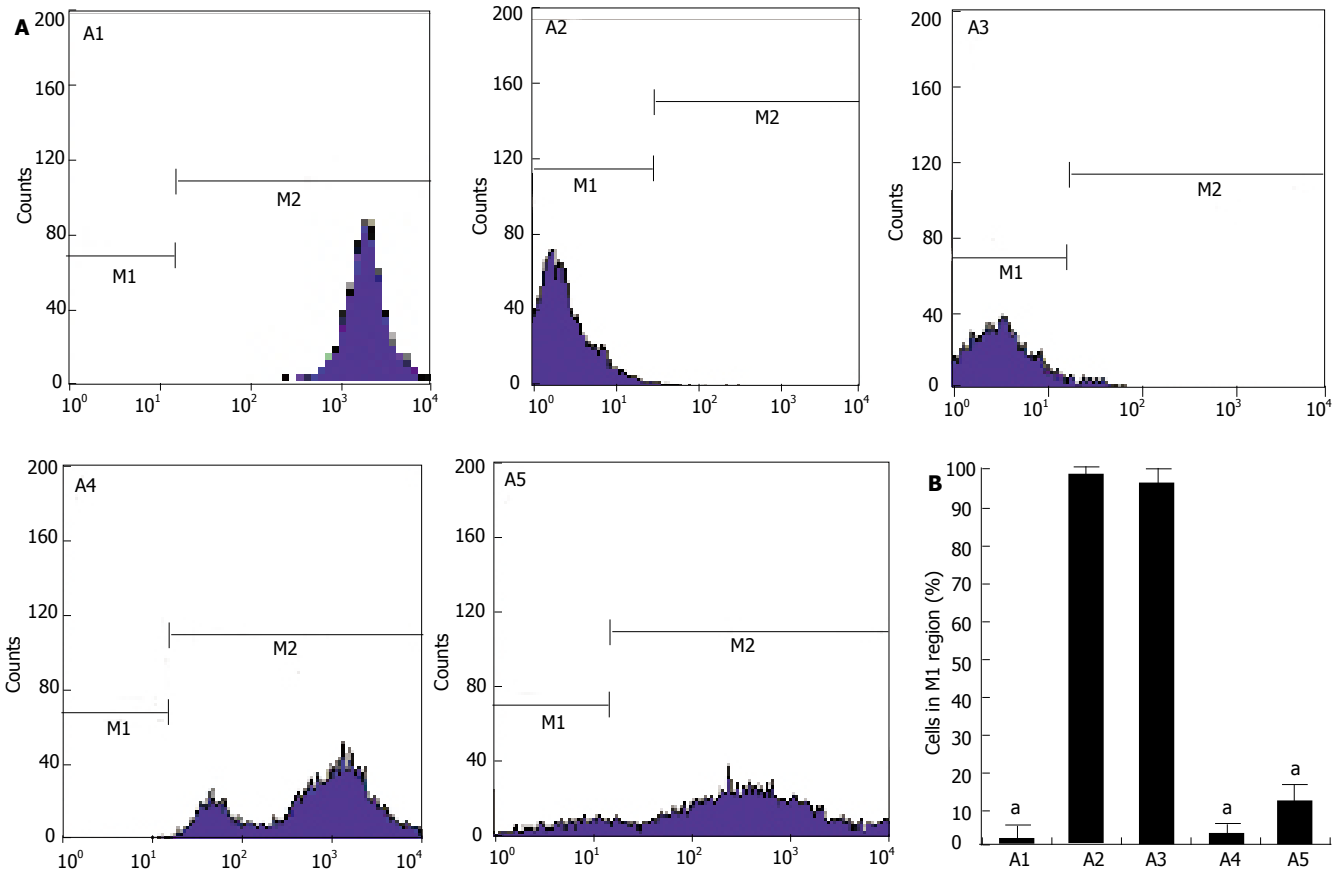

Figure 2 P-gp activity in HepG2 cells (A1), HepG2/ADM cells (A2), HepG2/ADM/vec control clone cells (A3); HepG2/ADM/pGenSil-l/MDR-B clone 1 cells (A4); HepG2/ADM/pGenSil-l /MDR-B clone 2 cells (A5) Rhodamine123 efflux was measured by counting cells in the M1 region of the plot. The marker bar M1 was set to indicate the cells with high Rhodamine123 efflux; the marker bar M2 was set to indicate the cells with low rhodamine123 efflux. Data were presented as mean ± SD. $^aP < 0.05$ *vs* untreated multidrug-resistant cell line HepG2/ADM.

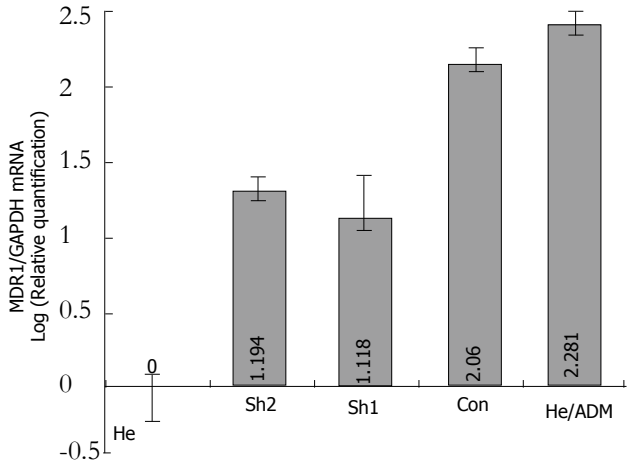

Figure 3 shRNA-mediated suppression of MDR1 mRNA. He, sh1, sh2, con and He/ADM represent HepG2, HepG2/ADM/pGenSil-l/MDR-B clone 1, HepG2/ADM/pGenSil-l/MDR-B clone 2, HepG2/ADM/vec control clone and HepG2/ADM, respectively. The relative expression values were the mean of triplicate real-time PCR reactions. Bars indicate SE.

using cell cytotoxicity to access adriamycin-specific ID50 levels. The resistance of HepG2/ADM to adriamycin was 102-fold stronger than that of the parental cell line at ID50 (Table 2). The HepG2/ADM/vec control clone had a similar resistance to HepG2/ADM cells. The resistance

Table 2 Effect of stable expression of shRNAs-mediated drug resistance to adriamycin

Cell line	Adriamycin IC50 (mg/L)	Resistance index (RI)
HepG2	0.141 ± 0.008	1
HepG2/ADM	14.394 ± 0.263[a]	102.085
Con	13.275 ± 0.353[a]	94.149
sh1	0.269 ± 0.035	1.908
sh 2	0.467 ± 0.617[a]	3.312

$^aP < 0.05$ *vs* ID50 value of HepG2. The data are presented as mean ± SD. Sh1, sh2 and con represent HepG2/ADM/pGenSil-l/MDR-B clone 1, clone 2, and the control HepG2/ADM/vec clone, respectively.

of HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 cells was decreased to a very low level near to that of the drug-sensitive HepG2 cells.

DISCUSSION

To improve the effect of RNAi-mediated gene silencing, some vector plasmids have been developed to express siRNAs as inverted repeats showing similar potency to initial RNAi. These siRNA-like molecules are commonly designated as shRNAs. For generating shRNAs, these vectors have been designed to be able to obtain the stable expression of shRNAs using strong RNA polymerase-II-depen-

dent promoters like CMV or EF1a, or RNA polymerse-III promoters like U6 or H1[10, 22-27]. Since experiences with antisense and ribozyme technology indicate that RNA polymerase-III expression systems can offer a great potency for maintaining stable expression of short RNA molecules *in vitro* as well as *in vivo*[28], the U6-driven expression vector pGenSil-1 was applied in this study. The size of the loop structure of shRNA has a considerable influence on the gene suppression activity. Brummelkamp *et al* [10] reported that a 9- nucleotide loop shows more effects than a 7-nucleotide loop, whereas a 5-nucleotide loop structure shows only moderate effects. Therefore, the two shRNAs were designed with a 9-nucleotide loop in our study.

The two shRNAs in this study showed different RNAi efficiencies. PGenSil-1/MDR-A was selected as previously described by Yague *et al* [17]. In his report, MDR-A shRNA constructed in pSUPER plasmid had an excellent RNAi effect on KD30 cell line, a multidrug resistant cell line derived from K562 leukemia cells. On the contrary, MDR-A shRNA did not show any significant RNAi effect on HepG2/ADM cells. One reason may be that the KD30 cell line is generated by a single-step selection in a low concentration of adriamycin (0.0174 mg/L)[17], while the HepG2/ADM cells are resistant to a high concentration of adriamycin. Another reason may be that the same siRNA sequence may have different RNAi efficiency in different tumor cells[12]. The different vector and loop structure of shRNA may also affect RNAi effects. Our study indicated that the pGenSil-1/MDR-B had a significant RNAi effect on HepG2/ADM cells. The MDR1 mRNAs were reduced by 93% and 92% respectively in HepG2/ADM/pGenSil-l/MDR-B clone 1 and clone 2 by expressions of MDR-B shRNA. The remaining MDR1 mRNA in the two clones was probably due to transgenic deletion, incomplete or inappropriate integration effects. The two clones showed a complete reversal of multidrug-resistant phenotype, indicating that the MDR-B siRNA might be very useful in designing new strategies for treatment of hepatoma in the future.

The empty pGenSil-l vector-transfected clone showed a slight decrease of MDR1 gene expression, which was suggested by real-time RT-PCR, Rh123 efflux test and cell cytotoxicity assay. That may be due to the withdrawal of adriamycin from the cell culture medium for a relatively long time[29].

The general obstacle to cancer gene therapy is the delivery of a therapeutic gene. Additional problems of clinical P-gp inhibition by anti-MDR1 siRNAs may arise from the physiological expression of this ABC transporter in several epithelial and endothelial cells. For example, P-gp is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain[30], suggesting that this efflux pump plays an important role in the blood-brain barrier and is crucial for limiting the potential neuro-toxicity of many anticancer drugs. Thus, in clinical practice the application of P-gp-inhibiting shRNA expression vectors should be restricted to P-gp expressing cancer cells. One promising strategy for cancer cell-specific delivery of siRNAs targeting MDR1 is to develop vector systems delivering siRNA in a cell-type specific manner. Lentiviral vectors could offer some advantages in upcoming preclinical studies[31]. A "replication-defective" E1A-mutant adenoviral vector that efficiently and selectively replicates in "classic" multidrug-resistant cells has been reported[32]. These viral vectors may contribute to developing more useful vectors for the MDR reversal gene therapy in multidrug-resistant cancers.

In conclusion, the highly adriamycin-resistant HepG2/ADM cells can be completely reversed to an adriamycin-sensitive phenotype by the shRNA-mediated RNAi. The target sequence can be integrated into gene therapy vectors for the potential application in clinic.

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