Article
Chinese Science Bulletin
Oncology
January 2012 Vol.57 No.1: 90–97
doi: 10.1007/s11434-011-4840-1

Knockdown of *MRP4* by lentivirus-mediated siRNA improves sensitivity to adriamycin in adriamycin-resistant acute myeloid leukemia cells

LIU Bei[1,3], ZHAO Li[2], MA HaiZhen[3], ZHANG Wei[2] & JIN Yu[1,4*]

[1] *Institute of Genetics, School of Basic Medical Sciences, Lanzhou University, Lanzhou 730000, China;*
[2] *Central Laboratory, The First Affiliated Hospital, Lanzhou University, Lanzhou 730000, China;*
[3] *Department of Hematology, The First Affiliated Hospital, Lanzhou University, Lanzhou 730000, China;*
[4] *Medical School of Nanjing University, Nanjing 210093, China*

Received April 10, 2011; accepted June 20, 2011; published online December 2, 2011

Chemotherapy remains the standard treatment for acute myeloid leukemia; however, the emergence of drug resistance is a major hurdle in the successful treatment of leukemia. The expression of multidrug resistance-associated protein 4 (MRP4) induces resistance in the adriamycin-resistant acute myeloid leukemia cell line, K562/ADR. The aim of this study was to investigate whether knockdown of *MRP4* by lentivirus-mediated siRNA could improve the sensitivity of K562/ADR cells to adriamycin. Five lentivirus-mediated short hairpin RNAs (lv-shRNAs-MRP4) were designed to trigger the gene silencing RNA interference (RNAi) pathway. The efficiency of lentivirus-mediated siRNA infection into K562/ADR cells was determined using fluorescence microscopy to observe lentivirus-mediated GFP expression. MRP4 expression in infected K562/ADR cells was evaluated by real-time PCR and Western blot analysis. The MTS assay was used to measure cell viability and flow cytometry was used to measure apoptosis. The transfection efficiency of K562/ADR cells was over 80 percent. The gene silencing efficacy of lv-shRNA1-MRP4 was superior to the other constructs. Infection of K562/ADR cells with lv-shRNA1-MRP4 led to strong inhibition of MRP4 mRNA and protein expression. Combined treatment with lv-shRNA1-MRP4 and adriamycin decreased cell growth and increased apoptosis compared to treatment with lv-shRNA1-MRP4 or adriamycin alone. These data indicate that in K562/ADR cells MRP4 is involved in drug resistance mechanisms and that lentivirus-mediated knockdown of *MRP4* may enhance sensitivity to adriamycin.

acute myeloid leukemia, lentivirus, siRNA, *MRP4*, K562/ADR cells

Citation: Liu B, Zhao L, Ma H Z, et al. Knockdown of *MRP4* by lentivirus-mediated siRNA improves sensitivity to adriamycin in adriamycin-resistant acute myeloid leukemia cells. Chin Sci Bull, 2012, 57: 90–97, doi: 10.1007/s11434-011-4840-1

Acute myeloid leukemia (AML) is a clonal disorder, characterized by the accumulation of an immature cell population in the bone marrow and/or peripheral blood. Despite improvements over the last 30 years in the combined use of various cytarabine (Ara-C) and anthracycline agents, the overall prognosis for AML remains poor. A major issue in the treatment of AML is multidrug resistance (MDR) [1]. Therefore, reversal of multi-drug resistance in leukemia cells is important to effectively treat AML patients [2,3].

Multidrug resistance-associated protein (MRP) is an ATP-binding cassette (ABC) transporter protein that is responsible for the pump resistance in various cancer cells. Over-expression of ABC transporters is commonly seen in MDR. MRP4 is a member of the C subfamily of ABC transporters. The first functional properties of MRP4 were described in a human T-lymphoid cell line in 1999. MRP4 is noteworthy for a particularly broad substrate specificity, including antiviral, antibiotic, cardiovascular and cytotoxic agents and it is a new therapeutic target for the modulation of various pathophysiological signaling processes [4,5]. Studies have shown that high levels of MRP4 expression, but not of any other MRP gene, were significantly associated

*Corresponding author (email: jinyu@lzu.edu.cn)

© The Author(s) 2011. This article is published with open access at Springerlink.com

with poor outcome in neuroblastoma [6]. MRP4 was also identified as a marker for immature stem cells in acute leukemia patients [7]. Moreover, the expression level of *MRP4* in 6-mercaptopurine resistant CEM-MP5 leukemia cells was approximately 4-fold higher compared with that in parental CEM cells [8]. However, the importance of MRP4 in adriamycin-resistant acute myeloid leukemia cell lines is unknown. Our previous experiments have shown that MRP4 is over-expressed in K562/ADR cells relative to parental K562 cells (Figure 1). Thus, we determined whether knockdown of MRP4 using RNAi could reverse resistance and sensitize K562/ADR cells to adriamycin.

As with any cancer therapy, effective delivery of the therapeutic agent to the proper target is a rate limiting factor and this holds true for the RNA interference (RNAi) pathway [9]. RNAi strategies that have been employed in cancer gene silencing include delivery of double stranded RNA complexes (siRNA), the use of double stranded DNA vectors encoding short hairpin-containing RNA sequences (shRNA), or the use of viral vectors containing double-stranded DNA encoding shRNA sequences. Viral vectors are particularly attractive because they can induce long-term knockdown of gene transcripts *in vivo*. Thus, the use of RNAi in a stable viral vector system, such as the lentivirus, is a possible strategy for stable gene knockdown [10–12]. Studies have demonstrated that RNAi is a valid approach to counter cellular drug resistance mediated by ABC transporters [2] and that RNAi has therapeutic potential in leukemia cell lines [13].

Our results suggest that MRP4 is a candidate molecule for mediating adriamycin resistance in acute myeloid leukemia cells. In addition, lentiviral-mediated siRNA targeting MRP4 provides an excellent approach for delivering siRNA into K562/ADR cells and this approach may be used for gene therapy of AML to produce an anti-proliferative effect and apoptosis when combined with adriamycin.

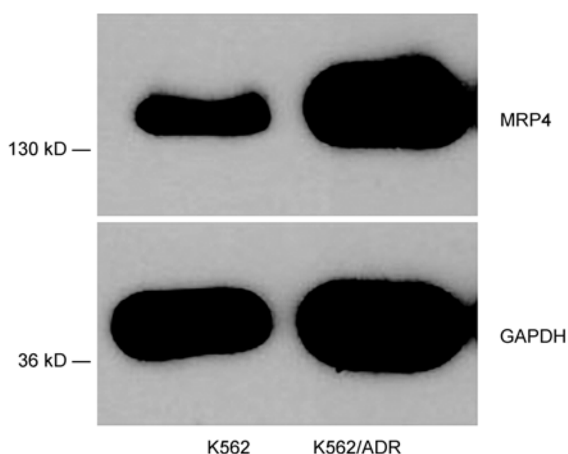

Figure 1 Western blot analysis of MRP4 levels. Compared to parental K562 cells, K562/ADR cells express a higher level of MRP4 protein. GAPDH, internal control protein.

1 Materials and methods

1.1 Drugs and cell culture

Adriamycin (Sigma, St.Louis, USA) was dissolved in distilled water and stored at –30°C until use. Adriamycin-selected human erythroid blast crisis chronic myeloid leukemia (K562/ADR) cells were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Parental K562 cells were provided by the Central Laboratory of the First Affiliated Hospital of Lanzhou University (Lanzhou, China). Cells were routinely maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/L *L*-glutamine, and 1% antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) under a humidified atmosphere of 5% CO_2 and air at 37°C. K562/ADR cells were incubated in adriamycin-free medium for over 1 week before experimentation. The IC_{50} value of K562/ADR was (60.33 ± 10.68) μg/mL.

1.2 Design of siRNA

Five MRP4-specific sequences (5′-CGTCCTTGTTAACTCTTCA-3′; 5′-CTGGTACTTAGGAATTTAT-3′; 5′-GGTTGCCTATGTGCTTCAA-3′; 5′-GAGCTGAGAATGACGCACA-3′; 5′-CTGGTACTTAGGAATTTAT-3′) were chosen according to general recommendations (http://www.ambion.com/techlib/misc/siRNA_finder.html) using MRP4 reference sequences (GenBank Accession No. NM_005845). The negative control siRNA sequence was 5′-TTCTCCGAACGTGTCACGT-3′, which was not homologous to any human DNA sequence. The siRNAs were designed and synthesized by GeneChem (Shanghai, China).

1.3 Construction of lentivirus-mediated short hairpin RNA (lv-shRNA)

The pGCL-GFP lentiviral vector containing a CMV driven GFP reporter and a U6 promoter upstream of cloning restriction sites (*Hpa* I and *Xho* I), which allow the introduction of oligonucleotides encoding shRNAs, has been previously described [14]. Sense and antisense oligonucleotides were designed for 5 targeting sequences (Table 1). The oligonucleotides were annealed and ligated into the *Hpa* I and *Xho* I sites of pGCL-GFP. Each shRNA consists of a T, a 21-nt sense sequence, a short spacer (TTCAAGAGA), the antisense sequence, 6 Ts (a stop signal for RNA polymerase III), and an *Xho* I site [15]. pGCL-GFP-shRNA-MRP4 constructs were then propagated and purified using a QIAfilter Plasmid Maxi Kit (Qiagen, Germany). Correct insertions of shRNA cassettes were confirmed by restriction mapping and direct DNA sequencing. The new engineered vectors were called lv-shRNA-MRP4; the negative control was named lv-shRNA-NC. The ability of the five lv-shRNA-MRP4 vectors to knock down MRP4 was investigated using

Table 1 shRNA template sequences

shRNA	5′	Stem	Loop	Stem	3′
shRNA1-antisense	Ccgg	taCGTCCTTGTTAACTCTTCA	TTCAAGAGA	TGAAGAGTTAACAAGGACGta	TTTTTg
shRNA1-sense	aattcaaaaa	taCGTCCTTGTTAACTCTTCA	TCTCTTGAA	TGAAGAGTTAACAAGGACGta	
shRNA2-antisense	Ccgg	aaCTGGTACTTAGGAATTTAT	TTCAAGAGA	ATAAATTCCTAAGTACCAGtt	TTTTTg
shRNA2-sense	aattcaaaaa	aaCTGGTACTTAGGAATTTAT	TCTCTTGAA	ATAAATTCCTAAGTACCAGtt	
shRNA3-antisense	Ccgg	caGGTTGCCTATGTGCTTCAA	TTCAAGAGA	TTGAAGCACATAGGCAACCtg	TTTTTg
shRNA3-sense	aattcaaaaa	caGGTTGCCTATGTGCTTCAA	TCTCTTGAA	TTGAAGCACATAGGCAACCtg	
shRNA4-antisense	Ccgg	aaGAGCTGAGAATGACGCACA	TTCAAGAGA	TGTGCGTCATTCTCAGCTCtt	TTTTTg
shRNA4-sense	aattcaaaaa	aaGAGCTGAGAATGACGCACA	TCTCTTGAA	TGTGCGTCATTCTCAGCTCtt	
shRNA5-antisense	Ccgg	aaCTGGTACTTAGGAATTTAT	TTCAAGAGA	ATAAATTCCTAAGTACCAGtt	TTTTTg
shRNA5-sense	aattcaaaaa	aaCTGGTACTTAGGAATTTAT	TCTCTTGAA	ATAAATTCCTAAGTACCAGtt	

real-time PCR.

1.4 Lentivirus production and concentration

Recombinant lentiviruses were produced by co-transfecting 293T cells with a lentivirus expression plasmid and a packaging plasmid using Lipofectamine 2000 [16–19]. Infectious lentiviruses were harvested 48 h post-transfection, centrifuged to remove cell debris, and then filtered through 0.45 μm cellulose acetate filters [15–18]. Virus titer was determined by fluorescence-activated cell sorting analysis of GFP positive 293T cells and was approximately 2×10^9 transducing units (TU)/mL medium.

1.5 Fluorescence microscopy

To determine the infection efficiency of K562/ADR cells with lv-shRNA-MRP4 and lv-shRNA-NC, cells expressing GFP protein were observed using fluorescence microscopy (Olympus, Tokyo, Japan) 5 d after transfection and images were captured. Successfully infected K562/ADR cells were collected for subsequent experiments.

1.6 Quantitative real-time RT-PCR

Cells were divided into 3 groups: control cells (CON, K562/ADR cells without transfection), lv-shRNA-NC cells (NC) and lv-shRNA-MRP4 cells (Knockdown cells, KD). Five days post-infection, total RNA was extracted from transfected K562/ADR cells with TRIzol (Invitrogen, California, USA). Reverse transcription (RT) was performed using a Reverse Transcriptase Kit (Promega, Bejing, China). *Actin* was used as an endogenous control. Real-time PCR was performed in triplicate using SYBR Mastermix on a TP800 (TaKaRa, Japan). Thermal cycling consisted of 95°C for 15 s, followed by 45 cycles at 95°C for 5 s and 60°C for 30 s. Values were normalized to the expression of the *actin* gene using the $2^{-\Delta\Delta C_t}$ method. The following primers were used: *MRP4*, forward, 5′-GTTCTTCTGGTGGCTCAAT-CC-3′; reverse, 5′-GGCTTCTGTGCGTCATTCTC-3′, fragment size 168 bp; *actin*, forward, 5′-GGCGGCACCA-CCATGTACCCT-3′; reverse, 5′-AGGGGCCGGACTCG-TCATACT-3′, fragment size 202 bp.

1.7 Western blot analysis

Cells were harvested 7 d after transfection and washed twice in PBS. Cell pellets were lysed by sonication in lysis buffer (100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 2% mercaptoethanol, 10% glycerol). The lysates were cleared by centrifugation and the supernatant protein concentration was measured using a BCA Protein Assay Kit (HyClone-Pierce, Nanjing, China). Total cell lysates (2 μg/μL) were boiled with SDS-sample buffer (100 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% mercaptoethanol and 0.4% bromphenol blue), separated on 8% sodium dodecyl sulfate (SDS)-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skimmed milk in PBS, membranes were probed with mouse anti-GAPDH (Santa Cruz, USA) or mouse anti-MRP4 (Abcam, Massachusetts, USA) antibodies followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies. Finally, proteins were detected using ECL Plus (Amersham, UK) and X-OMAT film (Kodak, USA).

1.8 MTS assay

Cell viability experiments were performed using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tet-razolium) dye reduction assay (Cell Titer 96_{Aqueous} One Solution Cell Proliferation Assay, Promega, China). Six groups of cells were assayed: control cells, lv-shRNA-NC cells, lv-shRNA-MRP4 cells (CON, NC, KD), with or without exposure to an IC_{50} dose (60 μg/mL) of adriamycin (CON+ADR, NC+ADR, KD+ADR). Cells (2×10^4 cells/well) were seeded in triplicate in 96-well plates and assays were performed at 24 and 48 h. At each time point, MTS solution was added to wells in a 1:5 ratio and allowed to incubate for 3 h at 37°C in 5% CO_2-humidified atmosphere. Wells were then analyzed with a plate reader to detect absorbance at 490 nm.

1.9 Flow cytometric apoptosis assay

The percentage of apoptotic cells was determined on a FACSCalibur flow cytometer (BD, New Iersey, USA). Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) analysis was performed according to the manufacturer's protocol (Annexin V-APC Apoptosis Detection Kit, eBioscience, USA). Approximately 1×10^5 cells/well were plated in triplicate in 6-well tissue culture plates. The same experimental groups used in the MTS assay were used in the flow cytometry assay. Cells were washed with PBS and resuspended in 100 μL Annexin V/PI incubation buffer and incubated for 15 min at room temperature in the dark. Binding buffer (400 μL) was then added to each sample and flow cytometry was performed.

1.10 Statistical analysis

All experiments were performed at least 3 times and statistical analysis was performed using the SPSS13.0 package (SPSS, Chicago, USA). Data are expressed as the mean ± SD. An ANOVA test was used for the comparison of more than 2 groups. *P* values of less than 0.05 ($P<0.05$) were considered to be statistically significant.

2 Results

2.1 Lentivirus-mediated high-efficiency infection of K562/ADR cells for knockdown of *MRP4*

The infection efficiency of K562/ADR cells was over 80 percent (Figure 2). Infected K562/ADR cells were analyzed by real-time PCR and Western blot analysis. Cell viability and apoptosis were determined by the MTS assay and flow cytometry, respectively.

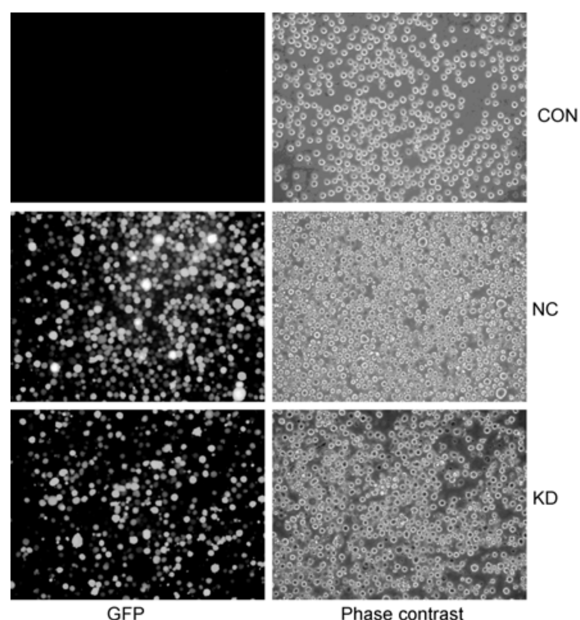

Figure 2 Fluorescence images of GFP and phase contrast images of the same fields. CON, K562/ADR cells without infection; NC, K562/ADR cells infected with lv-shRNA-NC; KD, K562/ADR cells infected with lv-shRNA-MRP4. Magnification: × 200.

2.2 Decrease of *MRP4* mRNA levels in human colon cancer RKO cells

We tested the efficiency and specificity of the 5 lv-shRNA-MRP4 vectors in RKO cells. Previous studies have shown that RKO cells express a high level of MRP4 (data not shown). Subconfluent RKO cells were infected with either lv-shRNA-NC or with each of the lv-shRNA-MRP4 vectors. Five days post-infection, infected RKO cells and control cells were collected and MRP4 expression was determined by real-time PCR. As shown in Figure 3, infection of all 5 lv-shRNAs-MRP4 vectors resulted in a considerable decrease in the levels of *MRP4* mRNA compared with that of control and lv-shRNA-NC cells ($P<0.05$), while the expression was similar between the control and lv-shRNA-NC cells ($P>0.05$). The gene silencing induced by lv-shRNA1-MRP4 was greater (>80%) than that of the other lv-shRNA-MRP4 vectors. Therefore, lv-shRNA1-MRP4 was used for the subsequent experiments in K562/ADR cells.

2.3 Inhibition of *MRP4* mRNA and protein expression in K562/ADR cells

To determine the effect of lv-shRNA1-MRP4 on the expression of *MRP4* in K562/ADR cells, mRNA and protein levels were analyzed. K562/ADR cells were infected with either lv-shRNA-NC or lv-shRNA1-MRP4. Five days post-infection, cells were collected and *MRP4* mRNA levels were detected by real-time PCR. Seven and 8 d post-infection, cells were collected and protein levels were detected by Western blot analysis. Lv-shRNA1-MRP4-infected cells displayed a remarkable reduction in *MRP4* mRNA levels (Figure 4(a)). The level of *MRP4* mRNA in lv-shRNA1-MRP4 cells (0.115±0.005) was lower than that of lv-shRNA-NC cells (1.001±0.051) and control cells

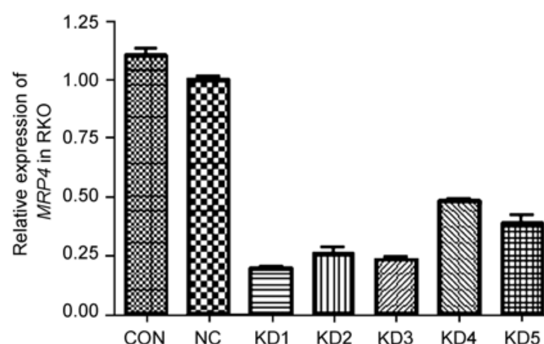

Figure 3 Decrease of *MRP4* mRNA levels in RKO cells detected by real-time PCR using the $2^{-\Delta\Delta C_t}$ method. CON, control cells; NC, lv-shRNA-NC cells; KD1, lv-shRNA1-MRP4 cells; KD2, lv-shRNA2-MRP4 cells; KD3, lv-shRNA3-MRP4 cells; KD4, lv-shRNA4-MRP4 cells; KD5, lv-shRNA5-MRP4 cells.

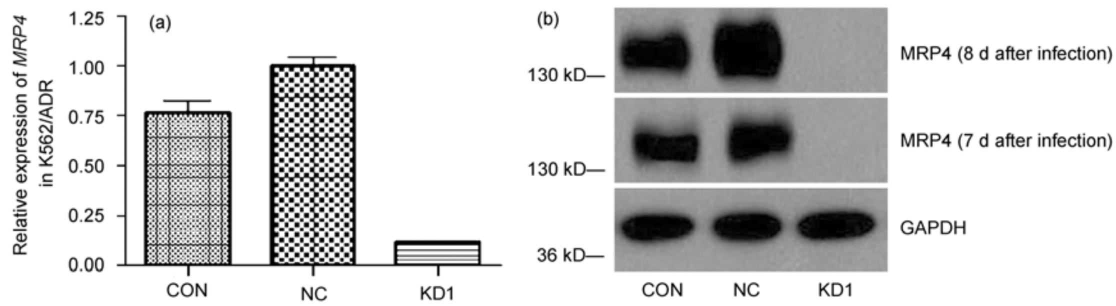

Figure 4 Reduction of *MRP4* mRNA and protein levels in K562/ADR cells. (a) Changes in *MRP4* mRNA levels detected by real-time PCR using the $2^{-\Delta\Delta C_t}$ method. (b) Knockdown of MRP4 protein, assayed by Western blot analysis. CON, control cells; NC, lv-shRNA-NC cells; KD1, lv-shRNA1-MRP4 cells; GAPDH, internal control protein.

(0.758±0.108) (P<0.05). Consistent with the reduced level of *MRP4* mRNA, lv-shRNA1-MRP4 infection resulted in a marked reduction of MRP4 protein levels at 7 and 8 d post-infection (Figure 4(b)). Western blot analysis demonstrated loss of MRP4 protein expression in K562/ADR cells treated with lv-shRNA1-MRP4. The MRP4 protein level was not affected in either the control or the lv-shRNA-NC-infected cells.

2.4 Inhibition of MRP4 increases cell death in K562/ADR cells

MTS cell viability assays were performed to determine whether knockdown of MRP4 increased K562/ADR cell death. Lv-shRNA1-MRP4, lv-shRNA-NC and control cells were assayed 24 and 48 h after an IC_{50} dose of adriamycin (Figure 5). The combination of lv-shRNA1-MRP4 and adriamycin had a significantly greater repressive effect on cell viability than each of the other treatments (P<0.05). That is, 48 h after infection, the survival of lv-shRNA1-MRP4, lv-shRNA-NC and control cells exposed to adriamycin was 47.7%, 76.3% and 79.1% (normalized to control cells), respectively. Lv-shRNA1-MRP4 or lv-shRNA-NC alone did not significantly inhibit cell proliferation compared to control cells (P>0.05).

2.5 Inhibition of MRP4 sensitizes K562/ADR cells to adriamycin-induced apoptosis

To explore whether loss of MRP4 resulted in increased adriamycin-induced apoptosis, K562/ADR cells were infected with lv-shRNA-NC or lv-shRNA1-MRP4. All cells (including control cells) were harvested for the assay 24 h after exposure to an IC_{50} dose of adriamycin, as described in Materials and methods. Annexin-V and PI staining were used to detect apoptotic cells, including cells in early-stage apoptosis (AV^+/PI^-) and late-stage apoptosis (AV^+/PI^+). As shown in Figure 6, the combination of lv-shRNA1-MRP4 infection and adriamycin exposure resulted in a significant increase in apoptosis as compared to each treatment alone (P<0.05). A significant increase in the number of apoptotic cells was observed in control, lv-shRNA-NC and lv-shRNA1-MRP4 cells treated with adriamycin (11.37%, 10.74% and 17.9%, respectively) as compared to control cells (8.03%), lv-shRNA-NC (7.94%) and lv-shRNA1-MRP4 (8.23%) cells without adriamycin treatment (P<0.05), while apoptosis was similar in control, lv-shRNA-NC and lv-shRNA1-MRP4 cells (P>0.05).

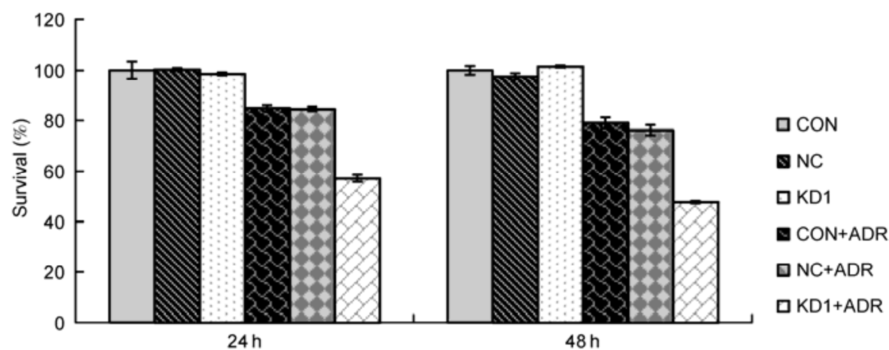

Figure 5 Inhibition of *MRP4* expression increases cell death in K562/ADR cells. MTS assays were performed to measure cell viability after *MRP4* knockdown and adriamycin treatment. K562/ADR cells were exposed to an IC_{50} dose of adriamycin 24 and 48 h after infection with lv-shRNA1-MRP4 or lv-shRNA-NC. CON, control cells; NC, lv-shRNA-NC cells; KD1, lv-shRNA1-MRP4 cells; CON+ADR, control cells treated with adriamycin; NC+ADR, lv-shRNA-NC cells treated with adriamycin; KD1+ADR, lv-shRNA1-MRP4 cells treated with adriamycin.

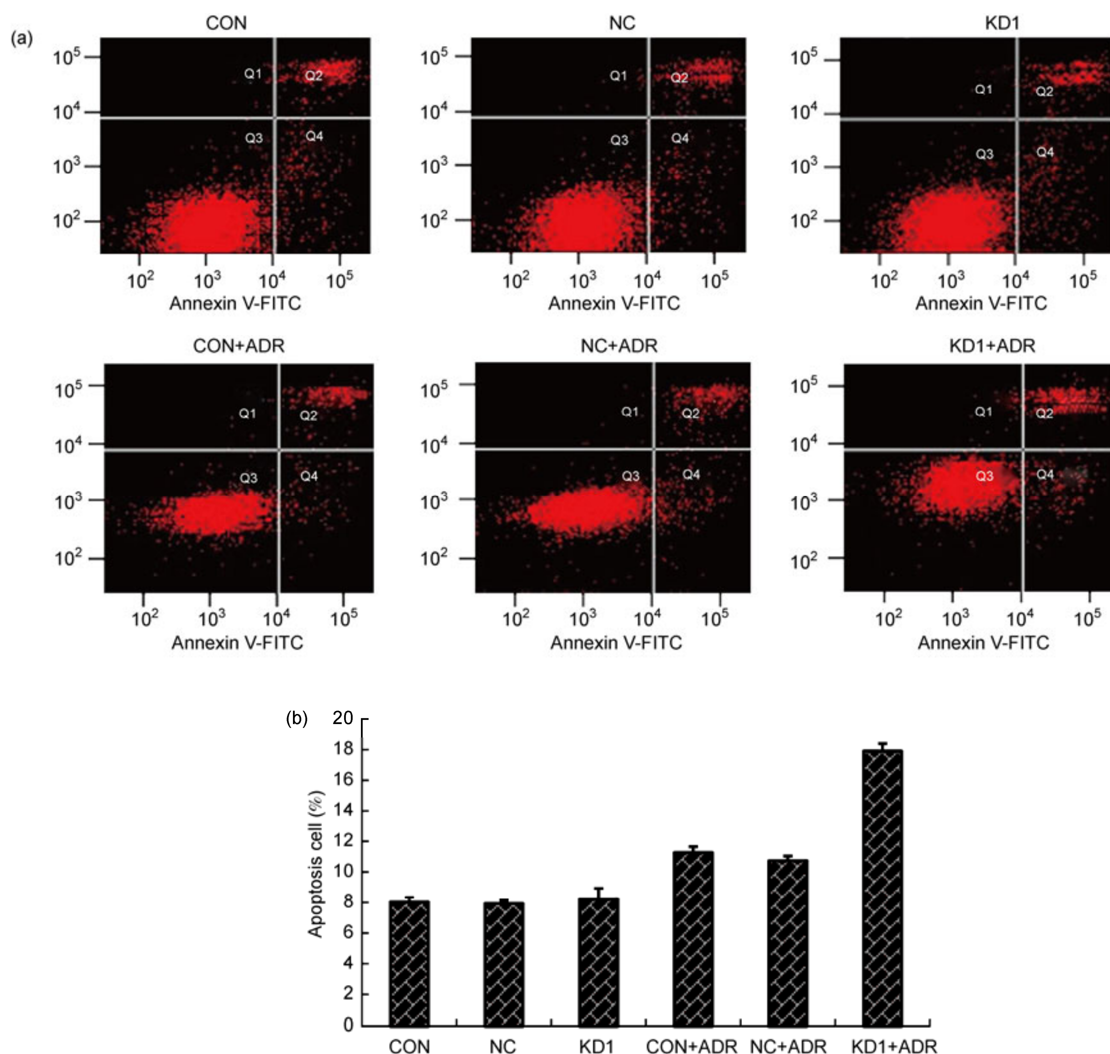

Figure 6 Inhibition of MRP4 sensitizes K562/ADR cells to apoptosis induced by adriamycin. (a) Cells were infected with lv-shRNA-NC or lv-shRNA1-MRP4 and treated with adriamycin for 24 h. Flow cytometric analysis after Annexin-V-PI staining was performed to evaluate the percentage of Annexin-V-PI positive cells. (b) Percentage of apoptotic cells, including both early-stage (AV^+/PI^-) and late-stage (AV^+/PI^+) apoptosis. CON, control cells; NC, lv-shRNA-NC cells; KD1, lv-shRNA1-MRP4 cells; CON+ADR, control cells treated with adriamycin; NC+ADR, lv-shRNA-NC cells treated with adriamycin; KD1+ADR, lv-shRNA1-MRP4 cells treated with adriamycin.

3 Discussion

Leukemia is one of the most common diseases in hematology. It shows frequent recurrence and a poor outcome despite combined chemotherapy because of the multi-drug resistance of leukemia cells [20–22]. Improving sensitivity of leukemia cells to chemotherapy is crucial for curing leukemia.

Adriamycin is a standard anthracycline anticancer agent for treating AML patients and is classified as a topoisomerase II inhibitor that induces cancer cells to undergo apoptosis by causing double-strand DNA breaks. Therefore, resistance to adriamycin in leukemia cells is a major obstacle to achieving treatment success. Adriamycin resistance may often result from over-expression of P-glycoprotein (P-gp), which is encoded by the multidrug resistance 1 gene (*MDR-1*). However, increased expression of MDR-1 does not always contribute to clinical resistance, at least in leukemia patients. The multifaceted nature of cellular resistance led to the discovery of the MRP genes. The MRP family is composed of 9-related ABC transporters that are able to transport structurally diverse lipophilic anions and that function as drug efflux pumps. In cultured cell lines, expression of various MRPs confers resistance to natural product anticancer drugs [23]. Studies have shown that MRPs may also contribute to anthracycline resistance in AML [3]. Furthermore, as yet unidentified MRP homologues that act as transporter proteins may play a role in MDR in AML. The presence of these nine transporters, and the finding that the lack of one transporter can be compensated for by the other transporters, may account for the somewhat disappointing results in some clinical studies using only P-gp inhibitors. MRP4 exports organic anions, including endogenous and exogenous substances, and is

involved in MDR [4]. Several studies have shown that MRP4 is over-expressed in neuroblastoma and in the CEM-MP5 leukemia cell line and MRP4 is also a marker for immature stem cells in leukemia patients. In addition, increased levels of MRP5 mRNA were observed in SBC-3/ADR, MCF7/ADR and K562/ADR cell lines as compared with the respective parental cells, and MRP5 is a candidate gene for adriamycin resistance in addition to MDR1 [24]. Amino acid sequence analysis has revealed that the nine transporters are composed of two nucleotide binding domains (NBDs) and at least two membrane spanning domains. MRP4, similar to MRP5, contains only two membrane spanning domains and is the most recently characterized transporter [25]. These data indicate that MRP4 may also be involved in resistance to adriamycin chemotherapy. Thus, we used K562/ADR cells as a model of drug-resistant leukemia because of the elevated expression of MRP4 as compared to the parental K562 cells. This observation suggests that MRP4 may be responsible for the adriamycin resistance in adriamycin-resistant acute myeloid leukemia cells.

SiRNA has become a powerful tool in molecular biology research [26]. Double stranded RNA (dsRNA) is processed into 21 to 28 nucleotide siRNAs, which are then incorporated into a complex that binds homologous target RNAs. The target RNAs then ultimately undergo cleavage leading to gene silencing. The sequence specific manner with which siRNAs bind mRNA has opened the door to the possibility of using this technique in a model of cancer therapeutics [27–29]. While direct use of siRNA is simple and results in functional gene silencing, its effect is transient. DNA-based RNAi has the potential of being stably introduced when used in a gene therapy context, allowing in principle, a single treatment of viral vector-delivered shRNA genes. Lentivirus vector-mediated gene transfer has some advantages for gene therapy: high efficiency of gene transfer into a wide variety of cells, including both dividing and non-dividing cells, long-term infection due to gene integration into the chromosome of host cells, no toxicity and no immune response [30–35]. Moreover, the RNAi pathway is functional in human myeloid leukemia cell lines, providing a means to overcome the resistance to chemotherapeutic agents and ultimately to augment the efficacy of chemotherapy in myeloid leukemia [13]. Therefore, we used lentivirus-mediated siRNA to study the functions of MRP4 in K562/ADR cells.

In the present study, we initially constructed 5 recombinant lv-shRNA-MRP4 plasmids. The lv-shRNA1-MRP4 plasmid was selected for subsequent experiments over the other four constructs because of its superior gene silencing efficacy; it could efficiently inhibit *MRP4* mRNA and protein expression in K562/ADR cells. Then, we determined cell viability using the MTS assay. The viability of K562/ADR cells was significantly reduced after combined lv-shRNA1-MRP4 infection and adriamycin treatment. To explore the mechanism of this reduced cell viability, flow cytometric analysis of apoptosis was performed. Knockdown of MRP4 by lv-shRNA1-MRP4 sensitized K562/ADR cells to adriamycin-induced apoptosis. Notably, a similar level of apoptosis was observed between the control, lv-shRNA-NC and lv-shRNA1-MRP4 cells, suggesting that silencing MRP4 alone did not induce apoptosis or reduce K562/ADR cell viability. These data indicate that reduced cell viability was due to apoptosis induced by adriamycin and that the combination of adriamycin treatment with MRP4 depletion by lv-shRNA1-MRP4 may be a potential approach to AML therapy. MRP4 is a membrane-embedded transporter molecule, which can act as an energy-dependent xenobiotic efflux pump [36], the transport activity of which results in decreased intracellular concentrations of anticancer drugs, including adriamycin. Thus, the specific inhibition of MRP4 by lentivirus-mediated siRNA results in the re-sensitization of K562/ADR cells to treatment with adriamycin.

It is known that glutathione (GSH) is involved in a number of biochemical processes and its levels in various cellular compartments are tightly controlled. The two primary mechanisms by which cells regulate intracellular GSH levels are by altering the rate of its biosynthesis and the rate of GSH export from cells. There is growing evidence that some of the MRPs are involved in GSH export and are major contributors to this process in cell models. Previous studies have demonstrated that drug-resistant cancer cells exhibit an elevated level of GSH compared to the drug-sensitive parental cells [37] and increased levels of a cisplatin-GSH conjugate have been detected in various cisplatin-resistant cell lines [38]. These data indicate that GSH may be associated with MDR in drug-resistant cancer cells. Supporting evidence has shown that GSH-conjugates are one of the main substrates for MRP4 [1]. It is not known whether the levels of cellular GSH are high or whether GSH is required for the transport of adriamycin in K562/ADR cells. In addition to its role as an efflux pump, MRP4 may contribute to adriamycin resistance in K562/ADR cells in other ways. Thus, further experiments are required to explore the function of MRP4 in these cells.

One potential pitfall of the present study was only focusing on the K562/ADR cell line, which is commonly used as an adriamycin-resistant leukemia model. Further observations with other cell lines would strengthen the significance of our study. Another potential problem is that endogenously expressed shRNA may induce a toxic effect [39]. Therefore, future studies will need to focus on the development of *in vivo* models.

In conclusion, we show for the first time that MRP4, in addition to MDR1, is likely to be involved in adriamycin resistance in K562/ADR cells. We successfully knocked down MRP4 in K562/ADR cells using lentivirus-mediated siRNA, which resulted in increased adriamycin-induced apoptosis and decreased cell viability, raising the possibility

that an appropriate combination of adriamycin application with MRP4 depletion may be a promising approach for the treatment of AML.

The authors thank the personnel of the Central Laboratory of the First Affiliated Hospital of Lanzhou University for technical assistance and Dr. Wang DeGui for insightful discussion and support. This work was supported by the Natural Science Foundation of Gansu Province (*20090501*).

1 Jäger W. Classical resistance mechanisms. Int J Clin Pharm Th, 2009, 47: 46–48
2 Modok S, Mellor H R, Chllaghan R. Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. Curr Opin Pharmacol, 2006, 6: 350–354
3 Van Der Kolk D M, De Vries E G E, Muller M, et al. The role of drug efflux pumps in acute myeloid leukemia. Leukemia Lymphoma, 2002, 43: 685–701
4 Ravna A W, Sager G. Molecular model of the outward facing state of the human multidrug resistance protein 4 (MRP4/ABCC4). Bioorg Med Chem Lett, 2008, 18: 3481–3483
5 Russel F G M, Koenderink G B, Masereeuw R. Multidrug resistance protein 4 (MRP4/ABCC4): A versatile efflux transporter for drugs and signalling molecules. Trends Pharmacol Sci, 2008, 29: 200–207
6 Murray D N, Smith J, Tanabe K, et al. Expression of multidrug transporter MRP4/ABCC4 is a marker of poor prognosis in neuroblastoma and confers resistance to irinotecan *in vitro*. Mol Cancer Ther, 2005, 4: 547–553
7 Steinbach D, Wittig S, Cario G, et al. The multidrug resistance-associated protein 3 (MRP3) is associated with a poor outcome in childhood ALL and may account for the worse prognosis in male patients and T-cell immunophenotype. Blood, 2003, 102: 4493–4498
8 Peng X X, Shi Z, Damaraju V L, et al. Up-regulation of MRP4 and down-regulation of influx transporters in human leukemic cells with acquired resistance to 6-mercaptopurine. Leukemia Res, 2008, 32: 799–809
9 Huynh T, Wälchli S, Sioud M. Transcriptional targeting of small interfering RNAs into cancer cells. Biochem Biophys Res Commun, 2006, 350: 854–859
10 Liao W, Ning G. Knockdown of apolipoprotein B, an atherogenic apolipoprotein, in HepG2 cells by lentivirus-mediated siRNA. Biochem Biophys Res Commun, 2006, 344: 478–483
11 Numnum T M, Makhija S, Lu B, et al. Improved anti-tumor therapy based upon infectivity-enhanced adenoviral delivery of RNA interference in ovarian carcinoma cell lines. Gynecol Oncol, 2008, 108: 34–41
12 Jung C R, Yoo J S, Jang Y J, et al. Adenovirus-mediated transfer of sirna against PTTG1 inhibits liver cancer cell growth *in vitro* and *in vivo*. Hepatology, 2006, 43: 1042–1052
13 Cioca D P, Aoki Y, Kiyosawa K. RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene Ther, 2003, 10: 125–133
14 Rubinson D A, Dillon C P, Kwiatkowski A V, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet, 2003, 33: 401–406
15 Brummelkamp T P, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science, 2002, 296: 550–553
16 Federico M. Lentivirus Gene Engineering Protocols (Methods in Molecular Biology). New Jersey: Humana Press, 2003
17 Tiscornia G, Singer O, Verma I M. Production and purification of lentiviral vectors. Nat Protoc, 2006, 1: 241–245
18 Sena-Esteves M, Tebbets J C, Steffens S, et al. Optimized large-scale production of high titer lentivirus vector pseudotypes. J Virol Methods, 2004, 122: 131–139
19 Reiser J. Production and concentration of pseudotyped HIV-1-based gene transfer vectors. Gene Ther, 2000, 7: 910–913
20 Kim D H, Lee N Y, Sung W J, et al. Multidrug resistance as a potential prognostic indicator in acute myeloid leukemia with normal karyotypes. Acta Haematol, 2005, 114: 78–83
21 Mi Y, Xue Y, Yu W, et al. Therapeutic experience of adult acute myeloid leukemia in a single institution of China and its relationship with chromosome karyotype. Leukemia Lymphoma, 2008, 49: 524–530
22 Koistinen P, Raty R, Itala M, et al. Long-term outcome of intensive chemotherapy for adults with de novo acute myeloid leukaemia (AML): The nationwide AML-92 study by the Finnish Leukaemia Group. Eur J Haematol, 2007, 78: 477–486
23 Kruh G D, Belinsky M G. The MRP family of drug efflux pumps. Oncogene, 2003, 22: 7537–7552
24 Yoshida M, Suzuki T, Komiya T, et al. Induction of MRP5 and SMRP mRNA by adriamycin exposure and its overexpression in human lung cancer cells resistant to adriamycin. Int J Cancer, 2001, 94: 432–437
25 Adachi M, Reid G, Schuetz J D. Therapeutic and biological importance of getting nucleotides out of cells: A case for the ABC transporters, MRP4 and 5. Adv Drug Deliver Rev, 2002, 54: 1333–1342
26 Wei X L, Ni H, Wang Q S, et al. Impact of *STAT4* gene silencing on the expression profile of proteins in EL-4 cells. Chin Sci Bull, 2009, 54: 3265–3270
27 Tomari Y, Zamore P D. Perspective: Machines for RNAi. Gene Dev, 2005, 19: 517–529
28 Doi N, Zenno S, Ueda R, et al. Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr Biol Jan, 2003, 13: 41–46
29 Choung S, Kim Y J, Kim S, et al. Chemical modification of siRNAs to improve serum stability without loss of efficacy. Biochem Biophys Res Commun, 2006, 342: 919–927
30 Andrew G, John A, Antigoni P, et al. Inhibiting primary effusion lymphoma by lentiviral vectors encoding short hairpin RNA. Blood, 2005, 105: 2510–2518
31 Cockrell A S, Kafri T. Gene delivery by lentivirus vectors. Mol Biotechnol, 2005, 105: 2510–2518
32 Sinn P L, Sauter S L, McCray Jr P B. Gene therapy progress and prospects: Development of improved lentiviral and retroviral vectors-design, biosafety, and production. Gene Ther, 2005, 12: 1089–1098
33 Li M J, James K, Li S, et al. Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, Anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. Mol Ther, 2005, 12: 900–909
34 Levine B L, Humeau L M, Boyer J, et al. Gene transfer in humans using a conditionally replicating lentiviral vector. Proc Natl Acad Sci USA, 2006, 103: 17372–17377
35 Li M, Rossi J J. Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. Methods Mol Biol, 2005, 309: 261–272
36 Dean M. ABC transporters, drug resistance, and cancer stem cells. J Mammary Gland Biol Neoplasia, 2009, 14: 3–9
37 Materna V, Liedert B, Thomale J, et al. Protection of platinum DNA adduct formation and reversal of cisplatin resistance by anti-MRP2 hammerhead ribozymes in human cancer cells. Int J Cancer, 2005, 115: 393–402
38 Materna V, Stege A, Surowiak P, et al. RNA interference-triggered reversal of ABCC2-dependent cisplatin resistance in human cancer cells. Biochem Biophys Res Commun, 2006, 348: 153–157
39 Chen T, Deng C S. Inhibitory effect of siRNA targeting survivin in gastric cancer MGC-803 cells. Int Immu, 2008, 8: 1006–1011

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.