RESEARCH ARTICLE

RNAi-based Knockdown of Multidrug Resistance-associated Protein 1 is Sufficient to Reverse Multidrug Resistance of Human Lung Cells

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Abstract

Up-regulation of multidrug resistance-associated protein 1 (MRP1) is regarded as one of the main causes for multidrug resistance (MDR) of tumor cells, leading to failure of chemotherapy-based treatment for a multitude of cancers. However, whether silencing the overexpressed MRP1 is sufficient to reverse MDR has yet to be validated. This study demonstrated that RNAi-based knockdown of MRP1 reversed the increased efflux ability and MDR efficiently. Two different short haipin RNAs (shRNAs) targeting MRP1 were designed and inserted into pSilence-2.1-neo. The shRNA recombinant plasmids were transfected into cis-dichlorodiamineplatinum-resistant A549 lung (A549/DDP) cells, and then shRNA expressing cell clones were collected and maintained. Real time PCR and immunofluorescence staining for MRP1 revealed a high silent efficiency of these two shRNAs. Functionally, shRNA-expressing cells showed increased rhodamine 123 retention in A549/DDP cells, indicating reduced efflux ability of tumor cells in the absence of MRP1. Consistently, MRP1-silent cells exhibited decreased resistance to 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and DDP, suggesting reversal of MDR in these tumor cells. Specifically, MRP1 knockdown increased the DDP-induced apoptosis of A549/DDP cells by increased trapping of their cell cycling in the G2 stage. Taken together, this study demonstrated that RNAi-based silencing of MRP1 is sufficient to reverse MDR in tumor cells, shedding light on possible novel clinical treatment of cancers.

Keywords: Multidrug resistance - MRP1 - ShRNA - protein expression - tumor cells

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Introduction

Lung cancer is one of the most common cancer diseases. A number of chemotherapeutic agents have been developed for the treatment of lung cancer, however, multidrug resistance (MDR) to cytotoxic drugs unavoidably impedes successful treatment in most of cases (Ye et al., 2009; Janet et al., 2013; Li et al., 2013; Kaewpiboon et al., 2014). Cancer cells with MDR are simultaneously resistant to drugs with different structures and cellular targets (Kamlesh et al., 2012; Chen et al., 2014). The dominant mechanism of MDR in a living cell lies in the active efflux of a broad range of anti-cancer drugs via the plasma membrane by MDR related proteins (Wu et al., 2010; Chen et al., 2011; Sun et al., 2013). Up-regulation of multidrug resistance-associated protein 1 (MRP1) is a well-defined phenotype during MDR (Pham et al., 2009; Yang et al., 2010; Ma et al., 2014). MRP1 belongs to a superfamily of ATP-binding cassette (ABC) transporters, members of which are associated with tumor resistance by increasing efflux ability and thus decreasing the intracellular accumulation of natural product anticancer drugs or other anticancer agents (Kepper., 2011). Overexpressed MRP1 has been identified as one of the main causes for MDR in several cancer cell lines originated from cancer patients (Cai et al., 2013; Wei et al., 2014). It was reported that that overexpression of MRP1 discovered in most Iranian pediatric leukemia patients at relaps (Frouzandeh et al., 2012). However, efficient approach to reverse MDR of tumor cells are still pendent.

RNA interference (RNAi) is a RNAi is currently used not only as a powerful tool in functional analyzing of a specific gene nucleic acid-based reverse genetic approach to silent target gene expression. or several specific genes, but also as a novel therapeutics (Shao et al., 2008; ketting., 2011; Felipe et al., 2014). Importantly, ShRNAs/siRNAs have been proved to be effective nucleic acid drugs for the treatment of many kinds of genetic or genetic curable diseases which have been considered as an important tool in molecular medicine. (Shao et al., 2010; Chen et

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al., 2010; Puthucode et al., 2012). With plasmid-based RNAi system, we tried to knockdown expression of MRP1, which restored the sensitivity of A549/DDP cells to DDP and MTT, mainly by decreasing the accumulation of exogenously drugs in these cells. RNAi-based silent of MRP1 provides an efficient approach for cancer treatments.

Materials and Methods

Plasmids

Two pairs of siRNAs, 5'-GGATCAAGACCGCTGTC AT-3' (1214–1234 nt) and 5'-AATTCTCAATGGGATCA AAGT-3' (1717-1737 nt), targeting to MRP1 (NM-004996), were designed according to the recommendation from the website http: //sirna.qiagen.com (Chen et al., 2006). BLAST research was performed to ensure the silent specificity of these shRNAs. The oligonucleotides were annealed and subcloned into the BamHI and HindIII sites of the vector Psilencer2.1-U6-neo to generate plasmids against MRP1 (P2.1-1 and P2.1-2). All constructs were verified by DNA sequencing.

Cell culture and transfection

A549, A549/DDP human lung cancer cell lines (established by Academy of Military Medical Science, Beijing, China) were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillinsodium, and 100 μg/ml of streptomycin sulfate at 37°C in a humidified atmosphere of 5% CO_2. To maintain the MDR probability, A549/DDP cells were cultured in 1.0 ug/ml DDP containing RPMI1640 medium. The cells were routinely diluted every 3 days.

A549/DDP multidrug-resistant human lung cell line was transfected with P2.1-1, P2.1-2, or empty vector P2.1 using eletroporation. The electric voltage of eletroporation apparatus was set at voltage 180V, implulse time 30 ms. 48h after transfection, cells were cultured in 10 cm cell plates with 700 ug/ml G418 containing medium. Concentration of G418 was reduced to 350ug/ml after 7 days culture. 2 weeks later, visible colonies were picked up and expanded. Stable expression was examined 9 weeks after transfection.

RNA extraction and quantitative PCR

Total RNA was extracted from transfected cells, treated with DNase I (New England BioLabs) and then reverse-transcribed with RevertAidTM first strand cDNA synthesis kit (K1621, MBI Fermentas). The primers used for detecting mrp1 gene expression were 5'-CGCTGAGTTCCTGCGTACC-3', and 5'-TCTGCGGTGCTGTTGTGG-3'. β-actin used as a normalization control was detected with primers 5'-TGACGTGGACATCCGCAAAG-3', and5'-CTGGAAGGTGGACAGCGAGG-3'.Quantitative real-time PCR was performed using Platinum® SYBR® Green qPCR SuperMix-UDG (11733-038, Invitrogen) on an (Bio, 7500) real-time PCR System (Liang et al., 2010).

Immunofluorescence analysis

Iimmunofluorescence was performed as previously described (Liang et al., 2004). Cells were cultured poly-L-lysine-coated (Sigma) coverslips for 24h and fixed with PBS containing 4% paraformaldehyde for 20 min. The fixed cells were permeabilized with 0.3% Triton X-100 in PBS containing 2% BSA for 5 min at room temperature. After blocking for 1h with 2% BSA in PBS, these cells were incubated with MRP1 monoclonal antibody 1: 200 diluted in 2% BSA containing PBS at room temperature for 3h and washed three timeswith blocking solution. Then, cells were incubated for 1h with secondary antibody of Alexa Fluor® 488 goat anti-mouse IgG (H+L) (A11029). After five washes in blocking solution and one wash in PBS, cells were mounted on slides with 1 mM PI containing 50% glycerol. A z-series of 1-μm optical sections was scanned through the 40× oil lens of a Zeiss 710 inverted confocal microscope. Images were processed by Zeiss LSM Image Browser (Version 3.0).

Intracellular Retention of Rhodamine123

The MRP1 activity was detected by efflux defect and thus the accumulation of intracellular Rhodamine123 (Rho 123). Living cells were incubated 1 h with 0.1 ug/ml Rho123 at 37°C. Then, the cells were extensively washed three times and re-suspended with ice-cold PBS at the cell density of 1×10^6 /ml .The fluorescence intensity of Rho123 was determined by a Flow cytometry (FCM, BD, FACsCalibur) combined with a 530 nm bandpass filter at an excitation wavelength of 488 nm.

Cytotoxicity Assay

Drug cytotoxicity was measured in vitro by pre-incubation with 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma) . Cells were plated in 96-well plates with a cell density of 2×10^3 per well, and cultured in medium containing increasing concentrations of DDP. After 48h of culture, 10 ul 5 mg/ml MTT was added to each well and the plates were incubated for 4h. The absorbance at 490 nm was read and the IC_{50} values were determined at three times. Relative reversal rate= (IC_{50A}-IC50B)/ (IC_{50A}-IC_{50C}), where IC_{50A} is IC_{50} values of non-transfected multi-drug resistanct cells, IC_{50B} is IC_{50} values of sensitive cells, IC_{50C} is IC_{50} values of transfected multi-drug resistanct cells. Survival data of transfected cells were evaluated by ANOVA for statistical significance.

Apoptosis assay

The transfected cells were cultured in 6-well plates. 24h later, DDP was included in the culture medium with a final concentration of 70 μM . After 48h treatment with DDP, cells were washed two times with PBS and fixed with 70% ice-cold ethanol for 16h. Cells were incubated with 1 ul PI at room temperature. Apoptosis was detected by FCM.

Statistical analysis

All experiments were replicated for at least three times. Statistical comparisons were performed with the two-tailed unpaired Student's t test, Kolmogorov-Smirnow test, or one-way ANOVA test as indicated. All tests were conducted using SPSS (Statistical Package for the Social

Sciences) 17.0. Significant differences were accepted at $p<0.05$.w

Results

Knockdown efficiency of MRP1

shRNA-based knockdown (KD) approach was used to silent expression of MRP1. Quantitative real-time PCR and western blot were performed to quantify the RNAi-mediated suppression of MRP1. The Relative mRNA level of MRP1 in A549 cells and A549/DDP cells with/without expressing of RMP1 shRNA or vector control were measured. MRP1 was efficiently silenced by both shRNAs, compared with that of A549/DDP control cells (Figture 1).

To assess the knockdown efficiency of MRP1 at proteins levels, we next performed immunofluorescence to quantify proteins expression of MRP1 in A549 cells, and A549/DDP cells with/without expressing MRP1 shRNA (Figure 2A). Compared with A549/DDP cells, MRP1 expression of shRNA expressing A549/DDP cells reduced by 83±1.20% and 48.16±0.69% respectively, while vec control cells showed no significant changes. (Figure 2B, C). These results suggest that expression of MRP1 in A549/DDP cells was efficiently silenced by both shRNAs.

MRP1 knockdown increased Rho123 retention

Since MRP1 functions to efflux exogenous chemicals from the cell body and thus is considered as the one of the main causes of MDR of tumor cells, we next investigated whether MRP1 knockdown increased the intracellular accumulation of Rho123 in A549/DDP cells. We found that intracellular Rho123 retention increased about 5-fold in shRNA expressing A549/DDP cells (A549/DDP, 15.93±0.58%; shMRP1-1, 84.02±0.59%; shMRP1-2, 82.56±1.37%) and showed no significant differences with that of DDP sensitive A549 cells (93.93±1.29%), while that in vector control-expressing cells remained largely unchanged (17.94±0.45%). These findings demonstrated that the increased efflux ability in tumor cells is mostly due to the up-regulation of MRP1, and that RNAi-based knockdown of MRP1 is an efficient approach to decrease efflux ability of tumor cells.

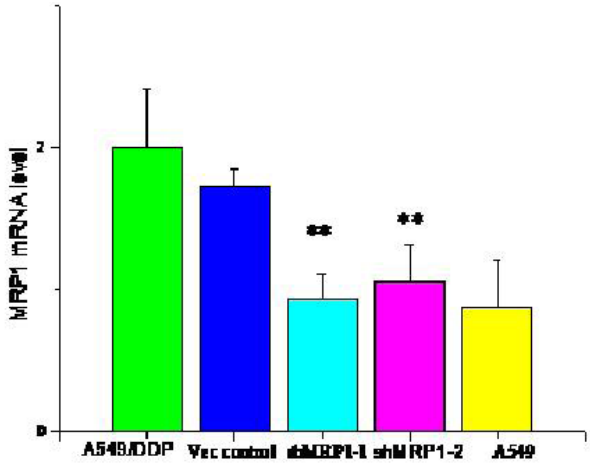

Figure 1. A Bar Graph Indicating MRP1 mRNA Level of Each Group. Data are presented as mean±s.e.m. ANOVA. $^{**}p<0.01$

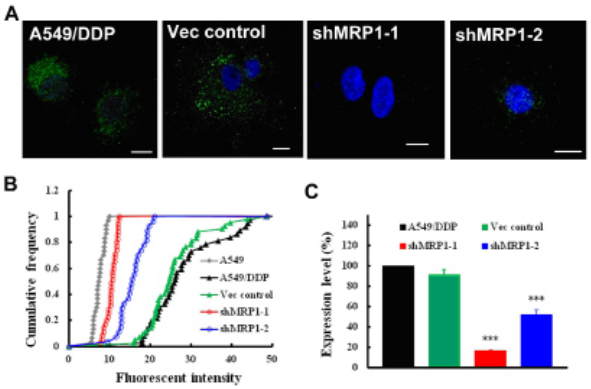

Figure 2. Immunostaining for Knockdown Efficiency of MRP1. (A) Representative immunofluorescent photomicrographs for expression of MRP1 in control and shRNA expressing A549/DDP cells Scale bars: 10 μm; **(B)** Cumulative frequency histograms MRP1 expression Kolmogorov-Smirnow test, $^{***}p<0.001$ for shRNA expressing *vs* control A549/DDP cells, and A549 vs A549/DDP cells; $p=0.518$ for A549/DDP cells vs that expressing vector control; **(C)** Quantification of relative MRP1 expression in control and shRNA expressing cells as in (A). Data are presented as mean±s.e.m. ANOVA. $^{***}p<0.001$

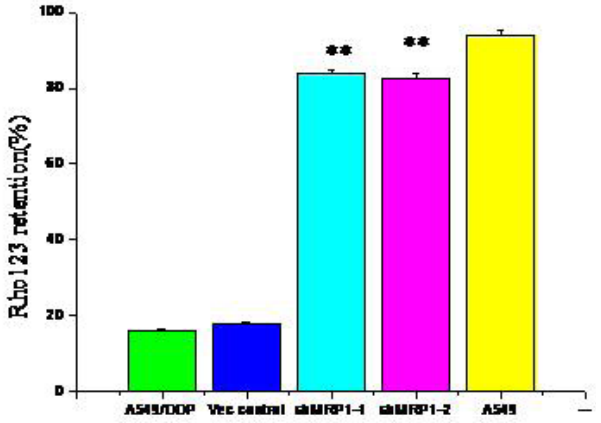

Figure 3. Accumulation of Rho123 in A549/DDP Cells $^{**}p<0.01$ for shRNA expressing vs control A549/DDP cells, and A549 vs A549/DDP cells; $p>0.5$ for A549/DDP cells *vs* that expressing vector control

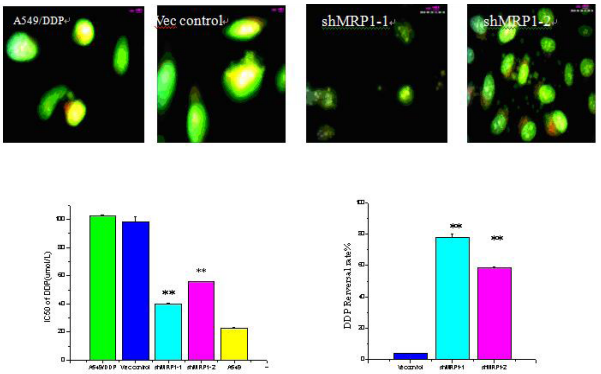

Figure 4. A dose-response curve of cells to DDP , IC_{50} and RI of MRP1 knockdown cells. $^{**}p<0.01$ for MRP1 knockdown cells of IC_{50} to DDP vs control A549/DDP cells, and A549 *vs* A549/DDP cells; $p>0.5$ for A549/DDP cells vs that expressing vector control. $^{**}p<0.01$ for MRP1 knockdown cells of DDP Reversal rate *vs* A549/DDP cells /vec contronl cells

Table 1. The Apoptotis Rate and Cell Cycle of the Cells Treated with 50μmol/L DDP for 48h

Groups	apoptotis rate (%) G0/G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
A549/DDP	0.73±0.12	68.78±1.15	20.19±0.56	10.74±0.91
A549/DDP/veccon	1.22±0.23	72.07±1.73	18.76±1.73	9.17±1.67
sh-2.1-1-1	8.61±0.58	73.90±0.58	2.46±0.58	22.84±1.73
sh-2.1-2-1	6.91±0.46	73.73±0.58	13.52±1.53	14.37±0.58
A549	10.92±0.51	71.62±3.46	2.55±1.20	25.83±0.16

**p<0.01 for MRP1 knockdown cells vs control A549/DDP cells, and A549 *vs* A549/DDP cells; p>0.5 for A549/DDP cells vs that expressing vector control

MRP1 knockdown decreased the cytotoxicity resistance

To investigate whether the decreased efflux ability in MRP1 knockdown cells reversed MDR of A549/DDP cells, we then performed MTT assay to examine the sensitization of these cells to DDP. All these cells showed a dose-dependent response to concentration of DDP, and IC50 of MRP1 knockdown cells decreased from 102.45±0.64 μM to 40.16±0.05 μM (shMRP1-1) and 55.72±0.36 μM (shMRP1-2) respectively. As a control, IC50 of blank vector expressing A549/DDP cells showed no significant differences. These results indicated that the up-regulated MRP1 expression take major responsible for MDR of tumor cells, and that MRP1 knockdown can effectively re-sensitize these tumor cells to multiple kinds of anticancer drugs.

MRP1 knockdown increased cell apoptosis

Since several anticancer drugs, including DDP, function to induce apoptosis of tumor cells during cancer treatment, we sought to determine the apoptotic rate of MRP1-knockdown A549/DDP cells with pre-incubation of DDP. Apoptosis was induced by pre-incubation with DDP, and consistently, apoptotic rate of A549/DDP cells increased about 8-fold in the absence of MRP1 (A549/DDP, 0.73±0.12%; shMRP1-1, 8.61±0.58%; shMRP1-2, 6.91±0.46%), similar with that of DDP sensitive A549 cells (10.92±0.51%). In contrast, apoptotic rate of of A549/DDP cells was largely unaffected by transfection with blank vector. This is consistent with the decreased efflux ability of A549/DDP cells with MRP1 knockdown. Specifically, the cell cycle of DDP treated cells were trapped in the G2 stage (Table1). Altogether, these results indicated that MRP1 knockdown decreased the efflux ability and thus reversed MDR of A549/DDP cells, bringing a bright future to the treatment of tumor diseases.

Discussion

Chemotherapy is currently one of the most important approaches for the systematic treatment of lung cancer. Up-regulation of MRP1 is identified as a main cause of MDR in cancer cells, leading to the frequent impediment for successful chemotherapy. However, an efficient approach to reverse MDR of tumor cells remains largely unknown. RNA interference represents a perspective strategy for down-regulation of the target gene, giving possibilities of its successful application in tumor disease treatment. Chun-Yu Zhu et al. (2011). observed that knock down of MDR1 by shRNA could increase the sensitivity to adriamycin in drug resistant gastric cancer cells.Here, we demonstrated that RNAi-based knockdown of MRP1 is sufficient to reverse MDR tumor cells and thus strengthening the treatment effect of chemotherapy (Palaniyandi et al., 2011).

The potent and fundamental gene-silencing mechanism of RNAi is to generate considerable excitement in the fields of molecular biology and gene therapy, but delivery is probably the biggest obstacle to the development of RNAi-based therapeutic agents (Xiao et al., 2008; Pecot et al., 2011). Vector-based delivery of shRNA could improve the efficiency of siRNA delivery which can obtain the stable expression of shRNAs using strong RNA polymerse-III promoters like U6 or H1 (Miyagishi et al., 2002; Ansaloni et al., 2010). Stable expression of shRNA targeting MRP1 in A549/DDP cells was firstly examined 9 weeks after transfection, and the higher knockdown efficiency of MRP1 was determined by quantitative real time PCR and immunofluorescence staining. Importantly, such RNAi-based higher silent efficiency could be sustained for more than 2 years, which makes it possible for the long-term effect of tumor diseases treatment.

MRP1 belongs to a superfamily of ATP-binding cassette (ABC) transporters, members of which are associated with tumor resistance by increasing efflux ability of exogenous anticancer drugs. Although the up-regulated MRP1 expression is a well-defined phenotype during MDR (Yang et al., 2010) whether silencing the overexpressed MRP1 is sufficient to reverse such MDR ability remains elusive. We fortunately discovered that MRP1 knockdown weakened the efflux ability, which was evidenced by the increased intracellular accumulation of Rho123 in A549/DDP cells. Surprisingly, the retention level of Rho123 in MRP1 knockdown cell was similar with that of DDP sensitive A549 cells. Consistently, the cytotoxicity resistant ability was also decreased in the absence of MRP1. These findings unambiguously suggest that the increased efflux ability in A549/DDP cells was largely contributed by the over-expressed MRP1, and that RNAi-based knockdown of MRP1 is sufficient to reverse MDR in tumor cells. Specifically, MRP1 knockdown accumulated the intracellular content of DDP and thus increased the apoptotic rat of A549/DDP cells by trapping their cell cycle in G2 stage.

Taken together, RNAi-based knockdown of MRP1 expression is sufficient to decreasing the efflux ability and thus reverse MDR in tumor cells, taking advantage for its potential application in clinic treatment.

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